RETINALDEHYDE AS VISUAL PIGMENT CHROMOPHORE

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R E T I N A L D E H Y D E

A S

V I S U A L P I G M E N T C H R O M O P H O R E

REDUCTION, ISOMERIZATION AND PIGMENT REGENERATION

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aan mijn Ouders,
Marianne en Tijs

C O N T E N T S

Our knowledge of the visual cycle is rather incomplete. Especially our knowledge of the fate of the chromophoric retinaldehyde leaves much to be desired. Already at the very first point of this cycle uncertainty exists. Although it is generally accepted that 11-cis retinaldehyde is the chromophoric group of rhodopsin, no direct proof for the presence of this isomer has yet been presented. We have therefore, attempted to extract the chromophore from native rhodopsin in as mild a manner as possible in order to achieve incontrovertible identification of its isomeric configuration (chapter 2).

When a rhodopsin molecule absorbs a photon, isomerization of the prosthetic retinaldehyde occurs. This conversion results in a series of non-photochemical reactions. The decay products have been characterized mainly by their spectra. Little is known about the binding of retinaldehyde in these photoproducts. We investigated the accessibility of the chromophoric site in photolyzed rhodopsin to exogenous 11-cis retinaldehyde, after fastening of endogenous retinaldehyde to opsin by $NaBH_4$-reduction. In this way we could determine whether or not a transiminization of retinaldehyde occurs during or after photolysis of rhodopsin. (chapter 3 and 4).

The role of retinoldehydrogenase in the visual cycle is still uncertain. Earlier studies in our laboratory have clearly demonstrated the presence of retinoldehydrogenase activity in rod outer segments, which in the presence of NADPH as coenzyme is capable not only of reducing free retinaldehyde, but also of hydrolyzing and reducing retinylidene aldimines to retinol. Purpose of our investigations on retinoldehydrogenase was to determine

1. whether structural factors favor the reduction of endogenous retinaldehyde over the reduction of exogenous substrate 2. how the substrate reaches the active site of the enzyme and 3. which geometrical isomer is the best substrate (chapter 5).

Under "normal" light conditions, photolysis and resynthesis should be in equilibrium, although the turnover of rhodopsin may be quite high. In this equilibrium a mechanism may be operating, which causes the reisomerization of retinaldehyde in the dark without migration of retinol to the pigment epithelium. We therefore searched for the presence of a system in the rod outer segments, which catalyzes the dark-isomerization of retinaldehyde (chapter 6).

CHAPTER 1

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE CATTLE RETINA

1.1 MORPHOLOGY

The vertebrate retina is a thin layer of tissue, lining the inside of the back of the eye. It consists of several layers, the pigment epithelium, the photoreceptor cells, the bipolar cells, the ganglion cells and the nerve fiber layer. The photoreceptor cells are found in the posterior region of the retina. As a consequence, the light has to pass several layers of the retina before it reaches the photoreceptor cells. The function of the photoreceptor cells is to convert light into an electrical signal, which is transmitted by the bipolar- and ganglion cells through the optic nerve to the brain.

Two types of photoreceptor cells are found in the vertebrate retina, the rods and the cones. The rods mediate twilight vision (scotopic vision), the cones function in bright light and mediate color vision (photopic vision). In the human retina only about 7% of the photoreceptors are cones. Towards the centre of the retina is a spot called the fovea, which is the fixation point of the eye. The fovea contains almost only cones. Each photoreceptor is composed of an inner segment, which is in close contact with the bipolar cells and an outer segment, which contains the visual pigment. The rod outer segments are cylindrical in shape and are connected to the inner segment by a cilium. At the earliest stage of development there is only a primitive cilium protruding from the inner segment. Through invagination of the

plasma membrane flat sacs are formed.

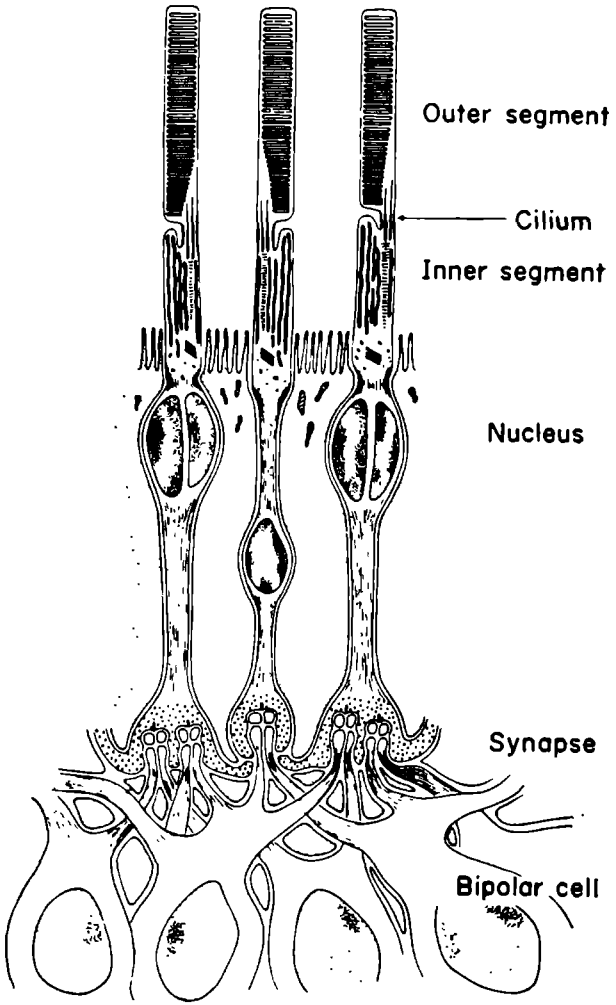


Fig.1 Structure of the vertebrate photoreceptor cell. From top to bottom: outer segment with rod sacs, inner segment with mitochondria, nucleus and synaps with bipolar cell. After Sjöstrand (1961).

At a later stage these invaginations pinch off and form separate disk-like sacs. The visual pigment forms an integral part of the rod sac membrane. In the mature rod a large pile (500 - 2000, depending on the species) of double membrane sacs of equal diameter is enclosed within the plasma membrane of the outer segment (fig. 1).

The mitochondria and ribosomes of the photoreceptor cell are located in the inner segment. Substances needed for the energy supply, the formation and the functioning of the outer segment must diffuse through the cilium to the outer segment.

1.2 THE VISUAL PIGMENT RHODOPSIN

1.2.1 Isolation and absorption spectrum

The rod or cone outer segments are characteristic structures, composed of membranes containing visual pigment molecules in a oriented position. The visual pigment rhodopsin, which is the main component of the disc membrane, is a protein with which phospholipids are associated. Rhodopsin can be solubilized and partly desintegrated from the rod sac membrane with aid of detergents. Digitonin has been used most frequently, but currently Triton-X-100, cetyltrimethylammonium-bromide (CTAB) and emulphogene are also used. In these detergents the membrane fragments are solubilized by incorporation into micelles. Dartnall (1961) showed that, although conditions are quite different from those in the living rod, the spectral properties of rhodopsin do not change upon solubilization. However other parameters, such as reaction kinetics, may change. For example, the pigment molecule in the membrane do not have much opportunity to collide with one another,

but this will be different in the solubilized state. Isolation of the pigment by means of detergents, although it does not alter the absorption spectrum, will surely alter properties like photosensitivity and reaction kinetics. Hence it is necessary to compile data from in vivo experiments and apply these to the interpretation of the data obtained from in vitro experiments. This is particularly the case for the reactions leading to resynthesis of bleached rhodopsin, the regeneration of the pigment.

The visual pigment rhodopsin is highly photosensitive. Therefore, the isolation procedure and all subsequent manipulations have to be carried out in dim red light, which does not cause photolysis of the pigment. Before isolation, it is customary to increase the rhodopsin content bij dark adapting the eye.

The absorption spectrum of solubilized rhodopsin shows major peaks at 278 and 500 nm.

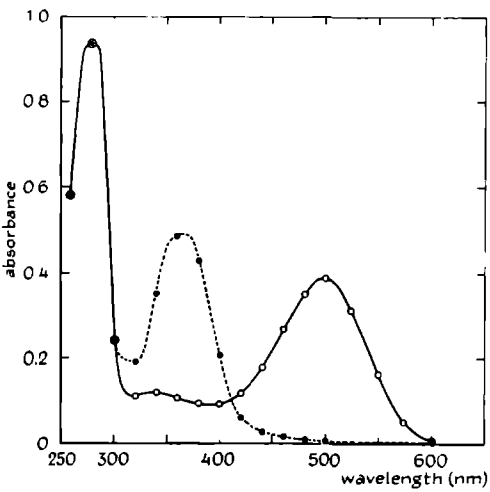


Fig.2 Absorption spectrum of rhodopsin in 1% digitonin, before (solid line) and after (dashed line) illumination in the presence of hydroxylamine.

The first peak originates from the aromatic amino acid residues in the protein, and shows virtually no change upon illumination. The 500 nm absorption peak is characteristic of the photopigment in the sense, that it disappears nearly completely upon illumination. Simultaneously, the absorbance at 360 nm increases (fig 2).

Several authors have reported an excellent agreement between the absorption spectrum of rhodopsin and the spectral sensitivity curve for scotopic vision (Lythgoe, 1937; Chase and Haig, 1939; Wald, 1945; Crescitelli and Dartnell, 1953), when the latter is corrected for absorption by lens and vitreous body. This means that light stimuli that are equally absorbed by rhodopsin, are equally seen. This is rather strong proof that the first step in scotopic vision is the absorption of light by rhodopsin.

The difference in absorbance at 500 nm before and after illumination can be used to calculate the molar concentration of visual pigment, when the molar absorbance is known. Furthermore, the absorption spectrum can be used as a tool to detect drastic changes in the environment of the chromophore, since these changes will abolish the absorption peak at 500 nm.

1.2.2 Orientation of the rhodopsin molecules

The molecular orientation of the pigment molecules in the outer segments has been investigated by means of polarized light (Schmidt, 1938; Denton, 1954 and 1959; Liebman, 1962; Wald, Brown and Gibbons, 1963). Spectra of single rods have been measured with polarized light passing through them axially and transversely. Plan-polarized light passing through the rod perpendicular to the long axis is maximally absorbed, when the

plane of polarization is rotated by 90°.This phenomenon is called dichroism, and it means that molecules of rhodopsin are oriented in the rod outer segment so that their chromophores lies perpendicular to the long axis of the rod and parallel to the disc plane. Within the disc plane the chromophoric groups are oriented randomly. The arrangement of chromophores is parallel to the electric vector of the wave motion of the physiological light stimulus, since light normally traverses the rods axially. This is the most favorable orientation for the absorption of light.

End-on dichroism of single axially oriented rod outer segments cannot be photoinduced by partial bleaching with plane polarized light. However, if a dark adapted frog retina is first fixed with glutaraldehyde and then partially bleached with polarized light, it becomes highly end-on dichroic (Brown, 1972). Transient end-on dichroism can be induced by a 5 nanosecond flash of polarized light; its rate of decay indicates a rotation time of 20 μseconds (Cone, 1972)

According to Denton (1959), the orientation of the retinol molecules formed after photolysis, is different. They require for maximal absorption, light with an electric vector parallel to the long axis of the rods. Upon photolysis the chromophore seems to come out of the disc membrane and gets oriented perpendicular to the disc surface. It might be that this orientation favors the migration of the hydrophobic retinol to other sites, through a layer of hydrophobic fatty acid side chains, belonging to the membrane phospholipids.

1.2.3 The chromophoric group

Wald (1933, 1934 and 1935) isolated from bleached rhodopsin a yellow pigment, which he called retinene. The remaining part of the visual pigment was called opsin. Morton and Goodwin (1944) and Ball, Goodwin and Morton

(1948) showed that this yellow pigment is the aldehyde of vitamin A. In this thesis the IUPAC names retinaldehyde and retinol are used for retinene and vitamin A, respectively (fig 3).

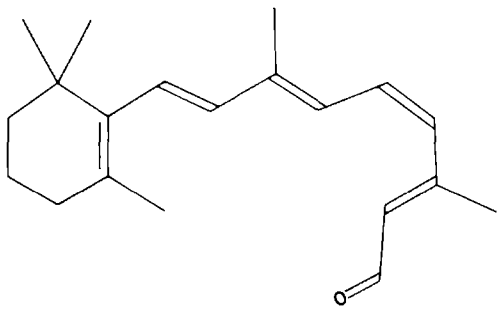
Incubation of opsin with retinaldehyde isolated from fish liver oil, led to the formation of rhodopsin (Hubbard and Wald, 1951), but no rhodopsin was formed when opsin was incubated with synthetic retinaldehyde (Hubbard and Wald, 1952). It is well known that carotenoids readily undergo cis-trans isomerization (Zechmeister, 1954). Hence it was rather logical to assume that the natural product contains a cis isomer of retinaldehyde which did not occur in the synthetic product.

Dieterle and Robeson (1954) prepared an isomer of retinaldehyde which reacts with opsin to give rhodopsin. The active isomer, which they called neo-b retinene, was obtained in crystalline form. Brown and Wald (1956) also obtained this isomer of retinaldehyde and the corresponding isomer of retinol. Irradiation of neo-b retinaldehyde gave a mixture of different isomers, similar to that produced by the action of light on the all-trans compound (Hubbard, Gregerman and Wald, 1953). The geometrical structure of the active neo-b isomer was, however, not known at that time.

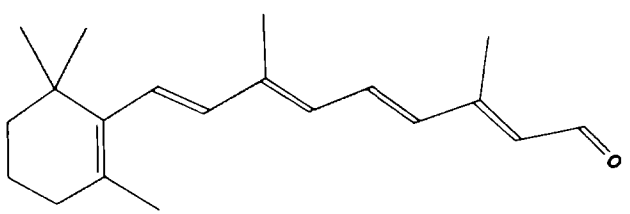
In a system of conjugated double bonds the thermodynamic stability depends to a great extent on the planarity of the system. The all-trans and the unhindered 9-cis, 13-cis and 9, 13-dicis isomers of retinaldehyde will be most stable. Overlap of the C_{10} hydrogen and the C_{13} methyl group leads to a torsion around the C_{11}-C_{12} bond. The geometry around the C_7-C_8 double is restricted too, because of steric hindrance between the ring and the side chain substituents. The existence of the 7 and 11-cis isomers was considered improbable, because steric

hindrance would cause the molecules to be nonplanar (Pauling, 1949).

It was,therefore,very surprising that after irradiation of an all-trans retinaldehyde solution not 4 but 5 isomers could be isolated (Robeson,Blum, Dieterle, Cawley en Baxter, 1955). The equilibrium mixture contained mostly the all-trans isomer and up to 25% neo-b retinaldehyde was found. Through direct synthesis the structure of the neo-b isomer was shown to be 11-cis retinaldehyde (Oroshnik, 1956; Oroshnik, Brown, Hubbard and Wald, 1956). Pauling's prediction about the non-existance of the 11-cis isomer was wrong.



11-*cis* retinal



trans retinal

Fig.3 Structure of the visual chromophores, 11-cis and all-trans retinaldehyde as determinded by X-ray analysis (Gilardi, Sperling, Karle and Karle, 1971). 11-cis retinaldehyde has a 12-s-cis conformation. The all-trans isomer displays a slight curvature.

Only recently, X-ray analysis has revealed the presice structure of 11-cis retinaldehyde (Gilardi, Sperling, Karle and Karle, 1971; fig 3). The 11-cis isomer was shown to be non-planar, despite the conjugated system of single and double bonds. Steric hindrance is relieved by rotation around the 12-13 single bond. This rotation was predicted by Honig and Karplus (1971) from calculations of torsional potentials of 11-cis retinaldehyde for rotation around the C_{12}-C_{13} bond.

11-cis retinaldehyde, bound to opsin, is very unstable in the light, but relatively stable in the dark. Free 11-cis retinaldehyde is stable when kept in the dark at low temperature.

1.2.4 Composition

Rhodopsin is a part of the lipoprotein structure that forms the rod sac membrane. The lipid composition of cattle rod outer segment membranes has been investigated by Poincelot and Zull (1969), Poincelot and Abrahamson (1970) and by Borggreven, Daemen and Bonting (1970). About 37% of the dry weight of washed bovine rod outer segments consists of protein, the remaining material consists mainly of lipids. Of these lipids 86% are phospholipids. Daemen, de Grip and Jansen (1972) showed that 87% of the insoluble protein in washed cattle rod outer segments is rhodopsin protein. Thus, rhodopsin is the principal component of the rod sac membrane. In these purified rhodopsin preparations at least two enzymatic properties (retinoldehydrogenase and Na-K-activated ATP-ase) are still present.

Hubbard (1954) calculated the molecular weight of rhodopsin from the difference in sedimentation rate of digitonin micelles and digitonin micelles containing

bovine rhodopsin. The value of 40,000 obtained in this way was called into question by the values of 27,000 - 28,600 determined from quantitative amino acid analysis (total protein weight per mole rhodopsin; Shields, Dinovo, Henriksen, Kimbel and Millar, 1967; Heller, 1968 and 1969; Shichi, Lewis, Irreverre and Stone, 1969). Recently Daemen, de Grip en Jansen (1972) found, using the same technique as well as polyacrylamide gel electrophoresis, a molecular weight of 39,100, which is much nearer to the original value of Hubbard. Column chromatografy was used for purification of rhodopsin from other membrane components (Heller, 1968; Shichi, 1969).

It is not yet clear, whether the phospholipids in the rod sac membrane are just structural elements of the membrane or have also functional properties. Borggreven, Rotmans, Daemen and Bonting (1971) were able to remove 95% of the phospholipids by phospholipase C treatment without damaging the spectral properties of the rhodopsin. The major part of the remaining phospholipids could be degraded by treatment with phospholipase A (Borggreven , Daemen en Bonting, 1972). Subsequent washings with a solution of bovine serum albumine led to removal of the lysophosphatides. The resulting preparation contained only 0.1 mole phosphatidylethanolamine and 0.1 mole phosphatidylserine per mole rhodopsin, but was spectrally intact. Hence, phospholipids are not involved in the binding of the chromophore nor do they influence the rhodopsin spectrum. There is,however, some evidence that phospholipids may play a role in the regeneration of rhodopsin after photolysis (Shichi, 1971; Zorn and Futterman, 1971).

1.2.5 Photolysis

When een rhodopsin molecule absorbs a photon, isomerization of the prosthetic group from the 11-cis to the all-trans configuration occurs (Hubbard and Wald, 1952). One of the results of this conversion is a marked loss in stability of the molecule, resulting in a series of non-photochemical (thermal) reactions. The first product, which has been detected after exposure of rhodopsin to light, is prelumirhodopsin (Yoshizawa and Kito, 1958; Yoshizawa and Wald, 1963). This product is converted very rapidly to lumirhodopsin (Hubbard, Brown and Kropf, 1959; Hubbard and Kropf, 1959; Kropf, Brown and Hubbard, 1959). At body temperature lumirhodopsin decays into metarhodopsin I (Wald, Durell and StGeorge, 1950). All these intermediates differ markedly in their absorption properties, but all contain all-trans retinaldehyde attached to the opsin moiety (Hubbard and Kropf, 1958; Grellman, Livingstone and Pratt, 1962; Matthews, Hubbard, Brown and Wald, 1963). Metarhodopsin I is somewhat more stable than prelumirhodopsin and lumirhodopsin. Metarhodopsin I and metarhodopsin II exist in an equilibrium in which higher temperature or acidity favor metarhodopsin II (Matthews, Hubbard, Brown and Wald, 1963; Ostroy, Erhardt and Abrahamson, 1966[a]). Metarhodopsin II has an absorption spectrum close to that of free retinaldehyde, but it contains the all-trans retinaldehyde still attached to a specific site on the opsin molecule.

Actual bleaching, a color change from red to near colorlessness occurs mainly between metarhodopsin I and metarhodopsin II. There are several reasons to assume that this conversion triggers visual excitation. First the decomposition of metarhodopsin II takes minutes,

while the interval between illumination and visual excitation is of the order of a millisecond, hence it is the last stage at which excitation can be caused. Secondly, the transition from metarhodopsin I to metarhodopsin II is the most drastic in the photolytic sequence: it is the step with the largest shift in the absorption maximum (478 to 380 nm), it is de first step requiring water, it involves uptake of protons and it is accompanied by a large entropy change. Apparently the molecule ondergoes a large conformational change, during which hitherto hidden hydrophylic groups of the protein become exposed to the surrounding medium.

At room temperature and physiological pH, the equilibrium is shifted nearly completely towards metarhodopsin II, which is also unstable under these conditions. Metarhodopsin II decays into a product which spectrally resembles metarhodopsin I. This product is called metarhodopsin III, pararhodopsin or transient orange (Weale, 1962; Ostroy, Erhardt and Abrahamson, 1966[a]; Frank and Dowling, 1968; Ebrey, 1968; Ripps and Weale, 1968 and 1969[b]) and is relatively stable in the living eye (Weale, 1967). Metarhodopsin III formation occurs parallel to the decay of metarhodopsin II into retinaldehyde (or retinol in vivo) and opsin (Matthews, Hubbard, Brown and Wald, 1963; Baumann, 1972).

Metarhodopsin III in turn is converted to retinylidene-opsin. The latter compound is not well characterized. For instance, it is not known whether the retinaldehyde is bound to one specific amino group or randomly bound to a variety of amino groups. The retinaldehyde binding site in retinylidene-opsin may be the active centre for reduction or re-isomerization. When conditions are unfavorable for such an enzymatic reaction, transiminization to other amino groups may occur.

Such products would not be spectrally distinguishable from the original retinylidene-opsin.

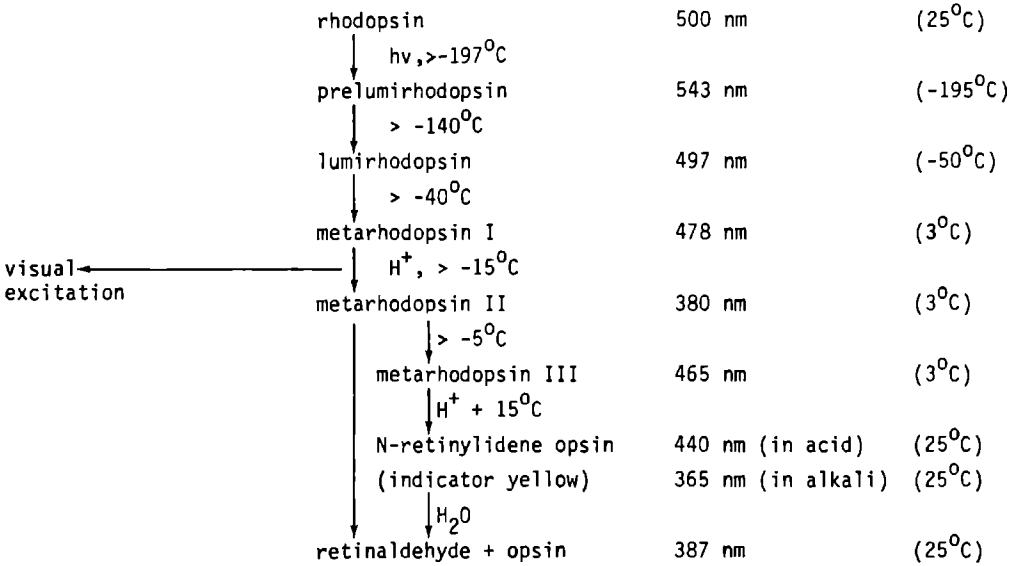


Fig.4 Thermal reactions following photolysis of bovine rhodopsin. Matthews, Hubbard, Brown and Wald (1963) reported a direct decay of metarhodopsin II in retinaldehyde and opsin, while the decay route according to Ostroy, Erhardt and Abrahamson (1966[a]) included metarhodopsin III (also known as pararhodopsin) and N-retinylidene opsin. In frog, metarhodopsin II decays both via metarhodopsin III and directly to retinaldehyde and opsin (Baumann, 1972). Figures in brackets are the temperatures at which the λ_m were measured.

In vitro retinylidene-opsin partially decays into free retinaldehyde and opsin. In vivo metarhodopsin III or retinylidene-opsin is reduced to retinol (Wald and Hubbard, 1949; Bliss, 1951[a]; Arden, 1954; Futterman and Saslaw, 1961; Bridges, 1962; Futterman, 1963; de Pont, Daemen and Bonting, 1970). The retinoldehydrogenase involved in this reaction is located in the outer segment of the visual cells. Futterman (1963 and 1965) has shown that NADPH is the functional coenzyme for this reduction.

All reactions following the effective absorption of a photon by rhodopsin are depicted in fig. 4.

1.2.6 Binding site

Retinaldehyde enters into combination with many compounds containing aminogroups to give aldimines:

$$R_1\text{-CHO} + R_2\text{-NH}_2 \longrightarrow H_2O + R_1\text{-CH=NR}_2 \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} R_1\text{-CH=}\overset{H}{\overset{|}{N}}\overset{|}{}\text{-}R_2^{+}$$

alkaline form acid form

aldehyde amine aldimine

Much work has been done to determine the binding site for the chromophoric group in rhodopsin. Collins (1953) and Morton and Pitt (1957) presented evidence for an aldimine bond between the chromophoric group and an aminogroup of opsin. Bleaching of rhodopsin in the presence of formaldehyde still results in retinylidene-opsin, although all free amino groups are blocked. Moreover, acid denaturation of rhodopsin results in acid retinylidene-opsin (λ max = 440 nm). In either experiment formation of a new aldimine link would seem to be impossible, so the aldimine link must already have been

present in the native rhodopsin. Strong support for the hypothesis that the 11-cis retinaldehyde is bound as a protonated aldimine came from Rimai, Kilponen en Gill (1970). They showed that the major line (1555 cm^{-1}) in Raman spectra of dark adapted bovine retinas has nearly the same wavelength as that in synthetic protonated retinylidene aldimines (1560 cm^{-1}), while the non- protonated form of the aldimines has a line at 1584 cm^{-1}.

Determination of the nature of the binding site is difficult, because the aldimine links is not stable against hydrolysis. Upon reduction to a C-N link the bond is stable during hydrolysis. Experiments have been carried out with the reducing agent sodiumborohydride ($NaBH_4$). Native rhodopsin in the dark does not react with this agent. Reduction occurs only after simultaneous illumination, and after hydrolysis the retinyl group was found linked to the ε-amino group of a lysine residue of the protein (Bownds and Wald, 1965; Bownds, 1967; Akhtar, Blosse and Dewhurst, 1965, 1967 and 1968; Akhtar and Hirtenstein, 1969).

The inability of native, dark adapted rhodopsin to react with sodiumborohydride is probably caused by protection of the chromophoric binding site by surrounding hydrophobic regions of the rhodopsin complex. This protection has been a major obstacle in studies on the chromophoric binding site in non-photolyzed rhodopsin. Reduction by sodiumborohydride of the labile aldimine bond to a secondary amine is only possible, when the linkage is made accessible to the surrounding medium by acid or alkaline denaturation (Poincelot, Millar, Kimbel and Abrahamson, 1970; Daemen, Jansen en Bonting, 1971). The conformational change of the rhodopsin complex during denaturation may, however,lead to transiminization of the chromophore. Such an artefactual transiminization

led Poincelot, Millar, Kimbel and Abrahamson (1970) to the erroneous conclusion that the chromophore is bound to the amino group of phosphatidylethanolamine in native rhodopsin. Transiminization can be avoided, when denaturation is performed with methanol- 0.1 M HCl, as shown by the study of model aldimines by Daemen, Jansen en Bonting (1971). After alkaline hydrolysis of rhodopsin, reduced and denatured in this way, these authors found retinyl-lysine in the hydrolysate. Another approach chemical modification of the free amino groups by methylacetimidate and displacement of the chromophore by a dansyl group, has recently confirmed lysine as the binding site of retinaldehyde in native rhodopsin (de Grip, Daemen and Bonting, 1973). From these experiments it can, however, not be concluded that in native rhodopsin and the photolyzed rhodopsin the retinaldehyde binding site is identical, since there are 14 lysine residues in opsin and there could occur a transiminization from one lysine residue to another during photolysis.

1.2.7 Conformational changes in opsin

Its link to opsin in native rhodopsin protects retinaldehyde from a variety of chemical attacks. For example it is not reduced by $NaBH_4$ (Bownds and Wald, 1965) and it is not oxidized and broken down by lipoxidase (Wald and Hubbard, 1960). It seems that the polyene chain is inaccessible even to small molecules. The 11-cis retinaldehyde is probably hidden in a hydrophobic pocket, which is formed by non-polar amino acid side chains of the opsin polypeptide chain.

The opsin molecule can assume at least two or three different configurations, one when it carries 11-cis retinaldehyde, another one when it is combined

with the all-trans isomer, and perhaps a third one when the chromophore is absent.

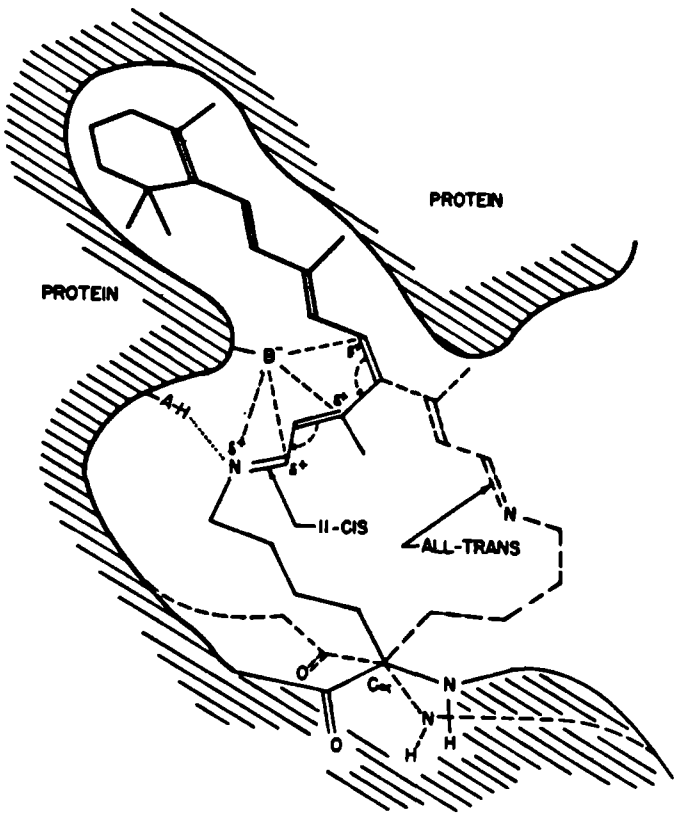


Fig.5 A diagramatic illustration of the binding of 11-cis retinaldehyde to a lysine residue in rhodopsin. Photolysis leads to isomerization of the retinaldehyde to the all-trans configuration (dashed line), which induces conformational changes in opsin, leading to initiation of the nerve impulse. According to Sundaralingam and Beddell (1972).

Opsin conformation appears to undergo important changes during photolysis. There is a small loss in absorbance at 280 nm and a much larger loss at 235 nm (Takagi, 1963). Such changes characterize changes in protein conformation, such as are observed in the unfolding of the secondary and tertiary structure of synthetic polypeptides and in protein denaturation (Wetlaufer, 1962). The optical rotatory dispersion (ORD) of cattle rhodopsin shows a maximum at 235 nm and decreases during photolysis (Crescitelli, Mommaerts and Shaw, 1966; Hubbard, Bownds and Yoshizawa, 1965; Kito and Takezaki, 1966). The ORD data seem to indicate that part of the rhodopsin protein chain is in the α-helix conformation (Schechter and Blout, 1964). Another indication for distinct differences in structure between opsin and rhodopsin is the fact that opsin is less stable than rhodopsin. Opsin is more sensitive to acid and alkaline denaturation (Radding and Wald, 1956[a], 1956[b]), it has also a lower heat stability (Hubbard, 1958), it has more reactive sulfhydryl groups (Wald and Brown, 1951; Ostroy, Erhardt and Abrahamson, 1966[b]) and acid binding groups (Radding and Wald, 1956[a]; Falk and Fatt, 1966[a] and 1966[b]). All these observations indicate a considerable conformational change at the bleaching of rhodopsin, which leads to a decrease in stability of the protein moiety. The fact that opsin is more readily denatured than rhodopsin, is probably the reason why vitamin A deficiency soon leads, not only to a loss of chromphore, but also of opsin (Dowling and Wald, 1958).

1.2.8 Visual excitation

Three hypotheses for the explanation of the visual excitation mechanism have been proposed;

the enzyme-hypothesis (Wald, 1956), the solid state hypothesis (Wald, Brown and Gibbons, 1963) and the ionic hypothesis (Bonting and Bangham, 1967). The arguments pro and contra these three hypotheses have been discussed at some length by Bonting (1969), who concludes in favor of the ionic hypothesis and sets forth a tentative explanation of the mechanism of excitation. Although recent electrophysiological work (Tomita, 1970; Hagins, Penn and Yoshikami, 1970; Zuckerman, 1971) and also our recent insights in the binding site of the chromophore in rhodopsin and metarhodopsin II (section 1.2.6) have made part of this explanation untenable, it is now virtually certain that an ionic mechanism is at the basis of excitation. The main problem left is how the bleaching of one or more molecules of rhodopsin in a rod sac membrane causes the decrease in Na^+-permeability of the outer membrane, demonstrated indepently by Tomita (1970) and Hagins, Penn and Yoshikami (1970). Lately Falk and Fatt (1972) have obtained evidence for an increased permeability of the rod sac membrane after illumination, as previously assumed by Bonting (1969). It may be that this effect is caused by the conformational changes in the opsin structure during photolysis. This permeability increase may lead to a release of Ca^{2+}-ions, which then cause the permeability decrease in the outer membrane (Yoshikami and Hagins, 1971).

1.2.9 Visual sensitivity and retinal photochemistry

The sensitivity of the eye depends greatly on the light intensity. Sensitivity is lost in the light and regained in the dark. The visual sensitivity (1/threshold) can be measured psychophysically in the human eye. In animal eyes the threshold of the b-wave

of the electroretinogram is used to measure the sensitivity (Dowling, 1960, 1963; Dowling and Hubbard, 1963; Baumann, 1967; Weinstein, Hobson and Dowling, 1967). Visual adaptation, the loss of sensitivity in light, covers a range of 9 - 10 log units. The rhodopsin concentration in man is measured in the experimental eye by retinal densitometry (Rushton, 1961[a], 1961[b]) and in animals in the control eye after extraction of the pigment (Dowling and Hubbard, 1963).

The time course of dark adaptation follows roughly the time course of regeneration of visual pigment (Wald, Brown and Smith, 1955; Wald, Brown and Kennedy, 1956), so it is obvious that there is some relation between the recovery of visual sensitivity and the regeneration of rhodopsin. However, als other factors must be involved, since there can be large changes in visual sensitivity without significant changes in pigment concentration (Wald and Clark, 1937; Rushton and Cohen, 1954; Weinstein, Hobson and Dowling, 1967, Baumann and Scheiber, 1968). This very rapid adaptation phase, which may be of neural origin, is followed by a more slow adaptation phase, which would be of a chemical nature (Dowling, 1963). In the isolated retina no regeneration occurs and only the "neural" adaptation phase is observed (Weinstein, Hobson and Dowling, 1967; Baumann, 1967). The recovery of sensitivity during the regeneration of visual pigment ("photochemical" adaptation) occurs in two stages, the early stage is associated with photopic vision, the later stage with scotopic vision. In other words,cone adaptation is faster than rod adaptation.

Various investigators have tried to determine the exact relation between adaptation and chemical changes in the retina. Until recently, it was generally

accepted that the log sensitivity is raised proportionally to the rate or the extent of pigment regeneration (Wald, Brown and Smith, 1955; Dowling and Wald, 1960; Rushton, 1961[b]). Baker en coworkers (Baker, Fulton and Rushton, 1969; Rushton, Fulton and Baker, 1969) showed that it was the fraction of bleached rhodopsin rather than the rate of regeneration, which determines visual adaptation.

Baumann (1967) suggested that a high retinol concentration in bleached isolated retinas might cause a decrease in visual sensitivity. Artificial removal of the retinol by wahing the retina with serum, leads to an increase in sensitivity. The effect of retinol migration after bleaching would be to keep the visual sensitivity at a higher level.

Donner and Reuter (1965, 1967 and 1968), however, found that the log threshold in frogs is raised initially by the amount of metarhodopsin II present, and in later stages by the log of the rate of rhodopsin regeneration. This conclusion has recently received wider support. Mainster and White (1972) showed that the metarhodopsin II concentration in rods is proportional to the threshold of the b-wave of the electroretinogram over the entire range of sensitivities. Separate mechanisms for a photochemical and a neural adaptation appear to be unnecessary. Rushton and Powell (1972) also suggest that the rise in threshold is entirely due to a photoproduct of rhodopsin, which rapidly decays and reaches an equilibrium with free opsin; this photoproduct is very likely metarhodopsin II.

1.3 THE VISUAL CYCLE

1.3.1. Introduction

Regeneration is a sequence of reactions resulting in the conversion of the all-trans retinaldehyde or the all-trans retinol derived from it, into the 11-cis isomer and its binding by opsin to form rhodopsin.

The rate, at which rhodopsin is bleached, is proportional to the intensity of the light and to the fraction of the light absorbed by rhodopsin. This means that the rate is nearly proportional to the product of intensity and rhodopsin density. The bleaching curve shows the expected exponential decay. The curve deviates, however, already after a few minutes from the theoretical exponential decay curve. There is an additional slowing down, presumably due to the onset of rhodopsin regeneration, which slows down the decay rate. After regeneration starts, the actual rate of change observed is the difference of bleaching and regeneration. Equilibrium will be reached, not when all the rhodopsin is bleached away, but when the rate of breakdown equals the rate of resynthesis. The regeneration curve can be measured separately from the bleaching curve by dark adapting the eye after a full bleach.

An important question is whether the regeneration mechanism in the light is the same as that in the dark. It could be that photoproducts cause a change in the regeneration rate. However, rat rhodopsin and human rhodopsin regenerate equally fast in the dark and in the light (Lewis, 1957; Rushton, 1958).

1.3.2 Regeneration in vivo

1.3.2.1 Methods

Changes in the eye during light and dark adaptation (bleaching and regeneration of rhodopsin) in living animals can be followed in two ways. In the first method animals are exposed to light or darkness for varying length of time and killed. From the excised eyes, the retina and the pigment layers are dissected. Rhodopsin and/or retinol compounds are extracted and determined spectrophotometrically. The second method is an in situ method, fundus reflectometry in which the light reflected by the fundus oculi is measured (Rushton, 1952; Weale, 1953[a] and 1953[b]). A monochromatic test beam of low intensity is sent into the eye, traverses the retina, is partly reflected at the back of the eye, traverses the retina again and emerges from the eye. Its intensity can be measured and will depend on the amount of pigment present in the pathway of the light. After illumination of the retina, the change in the test beam intensity reflects the change in photopigment, all other factors equal. An experimental difficulty is that since the fundus is lined by a black pigment epithelium, only about 1/20,000 to 1/10,000 of the incident light is returned in pigmented animals. Unwanted reflections and scattering make measurements at low intensity rather difficult. It is not possible with fundus reflectometry to measure simply and solely rod rhodopsin, but the best measurements are made 15^{o} temporal to the fovea in the so-called parafovea, which region is very rich in rods (Østerberg, 1935; Campbell and Rushton, 1955). Cone pigments can be measured in the fovea, which contain only cones.

Rushton, Campbell, Hagins and Brindley (1955)

proved that changes observed upon bleaching in the reflected light from the parafovea are due solely to changes in the rhodopsin concentration and not to other effects of illumination, because these changes satisfied the following criteria:

1. Bleaching and regeneration could easily be followed.
2. There were only changes in the part of the retina which had been bleached.
3. Bleaching was proportional to the product of time and intensity of illumination up to 50% bleaching and 45 sec of illumination (Campbell and Rushton, 1955).
4. Colored light of equal scotopic brightness bleaches equal amounts of rhodopsin.
5. The difference spectrum and the photosensitivity both show what might be expected of rhodopsin (Rushton, 1956).

1.3.2.2 Regeneration rates in various species

The animal used most frequently for regeneration studies are the rat and the frog. A few experiments have been done on rabbit and fishes. By means of retinal densitometry, Rushton and coworkers (Hagins and Rushton, 1953; Rushton, Campbell, Hagins and Brindley, 1955) have measured exactly the rhodopsin regeneration in the eye of the anaesthetized rabbit. The regeneration curve shows a linear rise for the first half hour, during which time 50 - 75% is regenerated. After that regeneration slows down. Then the curve clearly represents a first order reaction with a half-time of 10 min. Regeneration is complete in about 80 min.

Chemical determinations show that the regeneration

in the rat eye follows approximately the same course as in the rabbit eye, but is somewhat slower (Dowling and Hubbard, 1963; Dowling, 1960 and 1963). Half-regeneration is obtained after 30 min, while complete regeneration takes 100 - 120 min. In experiments of Lewis (1957), using the method of retinal densitometry, the time course of regeneration was remarkable slower, taking about 5 hrs for completion. Regeneration after complete bleaching was linear for 3 hrs. Thereafter the regeneration slowed down and followed an exponential course. The rather slow regeneration may have been caused by a poor condition of the animals. It appears that the entire course of regeneration in the rat eye can be described as a first order reaction (Tansley, 1931).

The time course of the rhodopsin regeneration in the frog eye is described by a sigmoid curve (Zewi, 1939; Peskin, 1942; Reuter, 1964[a] and 1966). The experimental results of these authors agree very closely. First, there is an initial delay in rhodopsin regeneration; then there is a linear rise of the rhodopsin concentration, which slows down after 60 - 65% of the rhodopsin has been regenerated. This phase of the regeneration takes 60 min. Thereafter the curve describes a first order reaction of 22 min half-time. Regeneration is complete in 2 hrs at 20° C (see also Hubbard and Dowling, 1962). The time course of regeneration in excised frog eyes is the same as in the eyes of intact animals (Reuter, 1966).

1.3.2.3 Regeneration of human visual pigments

Regeneration in man has for obvious reasons been investigated only by means of retinal densitometry. Densitometry on the parafovea permits measurement of the

rhodopsin regeneration after a full bleach. The rhodopsin concentration was seen to return along an exponential curve (Rushton, Campbell, Hagins ans Brindley, 1955; Campbell and Rushton, 1955; Rushton, 1961) with a half-time of about 4 min. Regeneration was complete in 30 - 40 min. A slightly shorter half-time of 2.8 min was reported by Ripps and Weale (1969).

The ultimate step in rhodopsin regeneration must be the reaction between opsin and 11-cis retinaldehyde, which would show second order kinetics. However the observed regeneration is a first order reaction. So, one of the reacting compounds is in great excess or constant. The rate of rhodopsin resynthesis in the human eye is at any moment proportional to the amount of opsin. This seems to imply that the amount of 11-cis retinaldehyde in the rod outer segments is approximately constant during regeneration.

The regeneration of cone pigments has been studied in all-cone retinas (Wald, 1937; Wald, Brown and Smith, 1955), by retinal densitometry (Rushton, 1963[b], 1965[b], 1958[a]; Weale, 1959 and 1962) and by measurements of the early receptor potential (Goldstein, 1968, 1970; Taylor, 1969). In man regeneration after a steady full bleach lasting 2 - 3 min follows an exponential course with a time constant of 120 to 130 sec (Rushton, 1958[a], 1963[b], 1965[b]; Baker and Rushton, 1965). Half-time for regeneration was 1.5 to 2 min and full regeneration required about 8 min. Experiments of Weale (1959 and 1962) on human cone pigments show an even faster regeneration (full regeneration in 3 to 4 min). Thus, human cone pigments regenerate 4 to 10 times as fast as the human rod pigment rhodopsin.

From the equations describing the rates of bleaching and regeneration it is possible to develop a general

equation for human cone kinetics (Rushton and Henry, 1969):

$$-\frac{dy}{dt} = \frac{(1-y)I}{4.10^6} - \frac{y}{130}$$

In this equation y is the fraction of pigment bleached, t is the time is sec and I is the retinal illumination in trolands (white light). A recombination of cone opsin with 11-cis retinaldehyde (Wald, Brown and Smith, 1955) implies a constant level of the retinaldehyde isomer, since the regeneration obeys first order kinetics, just as is the case in rhodopsin regeneration.

The general equation for human cone pigment kinetics has been tested by Alpern, Maaseidvaag and Ohba (1971) by measuring the photolysis rate at equilibrium and the equilibrium rate of regeneration immediately after the bleaching light is turned off. The results show that after prolonged bleaches of varying intensities, the amount of unbleached pigment could always be correctly calculated from the general equation.

Another method of measuring the regeneration of cone pigments is determination of the amplitude of the early receptor potential (ERP), which is directly proportional to the amount of unbleached 580 nm cone pigment in the retina (Cone, 1964; Taylor, 1969; Cone and Cobbs, 1969; Goldstein, 1968 and 1970). Taylor found that regeneration of cone pigment in the living frog eye follows an exponential course with a half-time of 22 sec. Goldstein measured in the isolated frog retina an exponential regeneration with a half-return time of 5 - 6 min. This result is rather surprising, since in isolated frog retina, no regeneration of rod rhodopsin

is found. In other words, whereas the regeneration of the frog rod pigment requires the participation of the pigment epithelium, regeneration of the cone pigment does not. The regeneration rate is higher than the rate of frog rhodopsin regeneration in vivo (Reuter, 1966; Hubbard and Dowling, 1962), but slower than the regeneration of cone pigments in vivo (Taylor, 1969; half-time 22 sec) or in man (Rushton, 1963[b] and 1965[b]; Rushton and Henry, 1968; Weale, 1959: half-time less than 85 sec).

1.3.2.4 Chemistry of regeneration.

In frogs most of the retinol released upon complete bleaching appears to leave the retina (Wald, 1935), most of it being found as retinyl esters in the pigment epithelium (Hubbard and Colman, 1959; Hubbard and Dowling, 1962; Futterman and Andrews, 1964). This migration process has been studied in more detail by Dowling (1960). The rat is a very suitable animal for these experiments, since practically no retinol is found in the pigment epithelium in the dark adapted state.

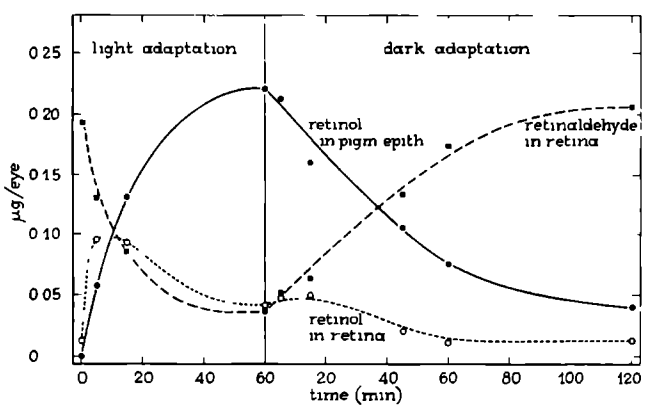


Fig.6 Distribution of retinaldehyde and retinol in the eye during light- and dark-adaptation. For a description see the text. According to Dowling (1960).

Rhodopsin binds 95% of the total retinol pool as 11-cis retinaldehyde. In this respect the rat is probably unique. Upon bleaching, the all-trans retinaldehyde is reduced in the retina to retinol and then migrates to the pigment epithelium (fig.6). In the fully light-adapted rat the pigment epithelium contains 80% of the total amount of retinol in the eye, and 92% of the retinol in the pigment epithelium is esterified. Krinsky (1958) has determined that in cattle 87% of the retinol in the pigment epithelium is esterified. In the rat the retinyl esters are all of the all-trans type. In frog and cattle a considerable part of the retinyl ester have the 11-cis configuration (42 and 65% respectively ; Hubbard and Dowling, 1962; Krinsky, 1958).

The 11-cis isomer is not found elsewhere in the body. It appears, therefore, to be formed in the eye itself, in cattle and frog probably both in the retina and in the pigment epithelium and in the rat in the retina only.

Since no isomerization occurs in the pigment epithelium of the rat, the purpose for the retinol exchange between the two tissues is not clear.

Hubbard and Dowling (1962) have studied the production and utilization of the 11-cis retinol, both in vivo and in vitro. They used frogs, because these animals store a considerable amount of retinol in their eyes. Light adaptation results in an increase of the amount of all-trans retinyl ester. No change is observed in the amount of 11-cis retinyl ester, although there is a continuous rhodopsin resynthesis in the light. This suggests that the rate of resynthesis is determined by a process which is slower than the isomerization process, e.g. migration of the retinol from the pigment epithelium to the retina. Alternatively, if isomerization occurs in the retina at

a rate sufficient to keep up with the bleaching, rhodopsin could be resynthesized without withdrawing 11-cis retinyl ester from the pigment epithelium.

The amount of 11-cis retinyl ester in the pigment epithelium diminishes during the course of regeneration, but the all-trans / 11-cis ratio remains unchanged. This means that the 11-cis isomer has not been drawn on preferentially, but that isomers of retinyl esters are used in about the same proportion as they are present in the light adapted eye. Hubbard and Dowling (1962) concluded that the isomerization of all-trans retinol ester is not the rate limiting step in the resynthesis of rhodopsin in the frog eye.

Only after completion of dark adaptation does the concentration of 11-cis retinyl esters increase slowly, reaching a maximal value of 42% after 24 hrs. This indicates the existence of a retinylester isomerase in the pigment epithelium.

Similar studies on the excised frog eye yield the same results as the in vivo experiments, only the amount of regenerated pigment is about 30 - 50% of the amount of rhodopsin resynthesized in vivo.

The existence of an enzyme, catalyzing the isomerization of all-trans retinyl ester into the 11-cis isomer, has also become likely from the work of Krinsky (1958) on the retinyl esters in cattle pigment epithelium. He found that 65% of the retinyl esters have the 11-cis configuration. A cell-free system from cattle eye tissues was capable of esterifying retinol. Some of this enzyme activity is located in the retina, but the pigment epithelium has a much higher activity of the esterifying enzyme.

In the rat retina NAD is predominantly in the oxidized form, while NADPH is largely in the reduced

form (Slater, Heath and Graymore, 1962). Therefore the oxidation of retinol might utilize NAD, while the reduction of retinaldehyde might use NADPH.

1.3.2.5 Photoregeneration

A photon absorbed by a rhodopsin molecule, causes photoisomerization of its prosthetic group, leading to a conversion of the rhodopsin into unstable intermediary photoproducts and finally to the stable retinol. The absorption spectra of the photoproducts and the spectral composition of the bleaching light determine whether or not photoisomerization of the all-trans retinaldehyde in these photoproducts will occur. Photoisomerization of the all-trans retinaldehyde chromophore is therefore possible in the prelumi-, lumi- and metarhodopsins.

Photoregeneration can only occur, if sufficient amounts of these intermediates can absorb light before they break down. This condition can be fullfilled by bleaching at very low temperatures, where intermediates are more stable, (Kito, Ishigami and Yoshizawa, 1961), or by using intense short flashes to bleach the pigment (Bridges, 1962; Hagins, 1956 and Rushton, 1964[a]).

After bleaching at very low temperatures only prelumi- and lumirhodopsin are formed, whose absorption spectra are similar to that of rhodopsin. These intermediates will absorb quanta of the bleaching light and some isomerization will occur. In solution, photoisomerization leads to equal amounts of the original 11-cis configuration and the 9-cis isomer. Since the rates of bleaching and photoreversal are about equal, 50% of the pigment is bleached and 50% is a 1 : 1 mixture of rhodopsin and isorhodopsin (Hagins, 1955; Bridges, 1962; Hubbard and Kropf, 1968; Collins ans Morton, 1950).

Photoisomerization of coloured intermediates could also be demonstrated during flash irradiation of rod suspensions (Bridges, 1962) and in the living rabbit or rat eye (Hagins, 1955; Dowling and Hubbard, 1963).

Flash photolysis of human cone pigment also yields photoisomerization (Rushton, 1963[c], 1964[a]). At low intensities there is no difference in bleaching between flashes of 0.2 msec and 10 sec duration. But at higher intensities the 10 sec flash bleaches far more rhodopsin than the 0.2 msec flash of equal intensity. These results are in good accordance with those obtained by Hubbard and Kropf (1958) on low temperature bleaching and those of Williams (1964) on flash bleaching at room temperature.

Flash bleaching of rhodopsin in the human retina, as performed by Ripps and Weale (1969) is different. An intense flash of 0.5 sec duration bleaches about 75% of the rhodopsin without reisomerization. The photodecomposition of rhodopsin in the human eye at body temperature probably goes too far in 0.5 msec to permit reisomerization.

During continuous illumination the amounts of the intermediates accumulating are so small that their isomerization into rhodopsin of isorhodopsin cannot be measured. Lewis (1957) found that prolonged bleaching with blue or green light does not accelerate the regeneration in the rat eye. Het concluded that the rate of regeneration during bleaching is the same as the regeneration in the dark. Rushton (1957) could not find any difference between rhodopsin contents in human eyes light adapted in matched blue and yellow lights, either during prolonged light adaptation(where the equilibrium levels are equal) or during the regeneration afterwards in the dark. There can be two causes for the failure of blue light to promote regeneration. One would be that the store of 11-cis retinol

is large enough to permit pigment regeneration from opsin for a long time. This is however very unlikely, since the 11-cis retinol reserve probably suffices for not more than a single replacement of rhodopsin. The more likely cause is that the concentration of all-trans retinaldehyde is too low to act as a substrate for the photoisomerization.

Thus it seems that photoregeneration cannot be the physiological mechanism for rhodopsin regeneration in the mammalian eye. Even the use of blue light, which is strongly absorbed by all-trans retinaldehyde, does not increase the yield of the 11-cis isomer and hence rhodopsin resynthesis. Hence, we must direct our attention to finding a system capable of isomerizing all-trans retinol or retinaldehyde to the 11-cis configuration in darkness.

Photoisomerization in cold-blooded animals may however be different, since thermal decomposition reactions are slower. Frank (1969), Baumann (1970) and Reuter (1964[a], 1966) found isorhodopsin in isolated frog retinas even under conditions of continuous exposure to normal light intensities. Reuter supposes that this exceptional photoregeneration is due to the greater stability of metarhodopsin in situ as compared to that in extracts (Dartnall, 1959, 1960, 1961); Denton, 1959; Bridges, 1962). Baumann (1970) saw no difference in regeneration after bleaching with either 450 or 500 nm light (both 23%), but less regeneration after bleaching with 540 nm light (9%); The regeneration spectrum showed that the substrate for regeneration has an absorption maximum at 380 nm, while the bleaching difference spectrum displays a maximum at 330 nm. These results indicate a regeneration from a substance that is different from the main product of bleaching. Most likely, the 330 nm absorbance is due to retinol and the 380 nm absorbance to retinaldehyde. There must at least be a minute amount of all-trans retinaldehyde present

in the isolated frog retina after a full bleach of 45 min duration. Possibly, the equilibrium of the retinoldehydrogenase reaction is not as far to the reduced side as it seems the case in the in vivo studies, in which retinol is removed by means of migration to the pigment epithelium. A few per cent retinaldehyde could be too small to be detectable in the bleaching spectrum, but enough to act as substrate for the regeneration. Conversion of the all-trans retinaldehyde clearly proceeds by means of photoisomerization, since the regeneration is found to be aided by light of shorter wave length. Decomposition of retinaldehyde by short wave length light causes a decrease in maximal regeneration. The combination of these two processes probably explains the fact that regeneration is equal after irradiation with 450 and 500 nm light, and only starts to decrease beyond these limits. The maximal regeneration that can be obtained seems to be 23%. The results of Baumann appear to be in conflict with those of Reuter (1966), who showed that light acts only by direct photoisomerization of metarhodopsin I and not by accelerating the isomerization of free retinaldehyde or retinol. Baumann, however, bleached the retina nearly completely and under such conditions Reuter found no preferential isomerization of metarhodopsin I.

When isolated frog retinas are exposed to a high intensity flash of very short duration, the photoregenerated material is found to be exclusively isorhodopsin (Baumann and Ernst, 1970). Whether or not isorhodopsin is a functional pigment in the frog retina is not clear.

1.3.2.6 Regeneration in excised eyes

The regeneration of visual pigment in excised eyes differs greatly in various species. No regeneration was found in the excised eye of the rabbit (Ayres and Kühne,1882). On the other hand, excised eyes of the frog (Kühne, 1878;

Hubbard and Dowling, 1962; Reuter, 1966) do regenerate. Light-adapted, excised frog eyes, having 90% of the rhodopsin bleached, regenerate 30 - 50% of the potential rhodopsin (Hubbard and Dowling, 1962).

The oxygen supply to the retina is probably an important factor in regeneration. The opened frog eye is able to use the atmospheric oxygen for its metabolic processes (Reuter, 1966). Replacing air by nitrogen decreases the regeneration capacity (Zewi, 1939). Lewis (1957) found that regeneration in rats with respiratory difficulties because of anaesthesia is slower than in normal rats.

1.3.2.7 Regeneration in isolated retinas

Thermal regeneration in isolated retinas is difficult to detect. Most work has been done on frog and rat retina (Crescitelli and Sickel, 1968). Perfusion of the isolated retina did not lead to regeneration of rod pigment in rat (Weinstein, Hobson and Dowling, 1967; Goldstein, 1967) and frog (Baumann, 1965).

No regeneration had been found in the isolated rat retina, until Cone and Brown (1969) showed that under special conditions regeneration in the isolated retina of the rat is also possible. The retina was isolated from the eye of dark adapted rats and was suspended in a small volume of Ringer solution in a small chamber, maintained at 37^{o}C. The volume of the chamber was only slightly greater than that of the retina. An area of 1 - 3 mm diameter was irradiated with 510 - 1000 nm light. Regeneration could be detected within minutes after the bleaching and was 80% complete within 3 hrs. At least three factors are essential for the observed regeneration, first the small volume of the chamber, secondly the temperature must be about 37^{o}C and thirdly the size of the bleached area must have a diameter not larger

than 1-3 mm. If the entire retina is bleached, there is only 40 to 50% regeneration.

The reisomerization mechanism for the regeneration of rhodopsin in isolated retina must be more labile than the corresponding mechanism for cone pigment regeneration, since little rhodopsin regenerates when the isolated frog retina is maintained under conditions, which still permit rapid regeneration of cone pigments (Goldstein, 1970).

1.3.3 Regeneration in vitro

The greater part of the rhodopsin cycle can be studied in vitro. The stage, at which one wants to examine rhodopsin regeneration, determines the experimental conditions and starting materials. The experiments can be carried out in the light or in darkness, incubating the photolyzed rhodopsin with either retinol or retinaldehyde, either the all-trans or the 11-cis form.

1.3.3.1 Photoregeneration

The first qualitive description of regeneration in solution was given by Ewald and Kühne (1878). Sixty years later the observation was made that the colour of the bleaching light could influence the amount of rhodopsin regenerated during subsequent incubation in the dark (Chase and Smith, 1939; Chase, 1937). Wald and Brown (1950) were able to regenerate rhodopsin after intensive bleaching with white light, when they added a very high concentration of retinaldehyde to the incubation mixture prior to bleaching. Hubbard and Kropf (1958) have shown that irradiation of photolytic products leads to partial regeneration of rhodopsin. Similar findings have been obtained by Wulff, Adams, Linschitz and Abrahamson (1958) and Williams (1964). Photoisomerization

of all-trans retinaldehyde, the endproduct of in vivo bleaching, to a mixture of 11-cis and 9-cis retinaldehyde, depends on the ability of the retinaldehyde to absorb the bleaching light. This light absorption is maximal at 360 nm, but at this wavelength considerable decomposition also occurs. Maximal photoregeneration is obtained after exposure of the rhodopsin solution to 440 nm light, which leads to 15 - 25% regeneration (Shichi, 1971).

1.3.3.2 Isomerase

When a bleached sample of rhodopsin is left in the dark without addition of cofactors, little rhodopsin resynthesis occurs and this can be accounted for by photoregeneration. This suggests that one or more substances promoting regeneration can be washed out of the preparation. Little is known about the localization of these cofactors, be they isomerase, NADPH or other cofactors. Neither do we know much about how 11-cis retinaldehyde is formed from all-trans retinaldehyde or all-trans retinol.

Hubbard (1956) has reported the presence of an enzymic factor, present in cattle retinas, which catalyzes in the light the isomerization of all-trans retinaldehyde to the 11-cis isomer. She doubted the significance of this enzyme, since regeneration is known to occurs in the dark also and the action of the enzyme was too slow to explain the observed regeneration rate in vivo. Moreover, retinaldehyde can only be isomerized by short wavelength light and this is poorly transmitted to the retina. Also, there will be very little free retinaldehyde in the retina, since most of it is reduced to retinol. Other evidence against the retinaldehyde isomerase is the proportion of 11-cis retinol stored in dark adapted cattle and frog eyes. This proportion far exceeds what one would expect from the dark equilibrium catalyzed by the

isomerase. If the isomerase would be involved, the resulting 11-cis isomer must be stabilized in some way, either by binding to a specific protein, or by selective esterification. There is no evidence that either process operates in the eye. An other pathway surely must exist.

1.3.3.3 Regeneration from retinaldehyde

The problem of reisomerization can be circumvented by incubating bleached rhodopsin with the required 11-cis isomer. Such experiments were performed by Hubbard and Wald (1952), who incubated five isomers of retinaldehyde with opsin. The 11-cis isomer only gave rhodopsin. The 9-cis isomer also reacts with opsin, but yields isorhodopsin (λ_m = 485 nm vs. 498 nm for rhodopsin). The reason for the ability of this isomer to react with opsin is that 9-cis retinaldehyde in shape very much resembles the 11-cis isomer. Isorhodopsin has not been found in the vertebrate retina under physiological conditions.

The reaction of opsin and 11-cis retinaldehyde follows a bimolecular course and no other factors (e.g. enzymes) are needed (Wald and Brown, 1956). The rate of the reaction between opsin and 11-cis retinaldehyde differs greatly from species to species. Alligator rhodopsin is synthesized more rapidly in solution than frog, cattle, rabbit and rat rhodopsin (Wald, Brown and Kennedy, 1956).

The synthesis of rhodopsin in vivo involves a chain of reactions in which the reaction of opsin and 11-cis retinaldehyde is only the last step. Therefore a direct comparison of the in vivo and the in vitro experiments is not permissible, since in vivo regeneration is greatly influenced by the rigid structure of the rods, in which opsin and retinaldehyde are in fixed positions and move in restricted ways. Steric and diffusional factors, present in vivo, may greatly influence reaction-kinetics.

1.3.3.4 Regeneration from retinol

The occurence of retinoldehydrogenase and the presence of retinol in light adapted animals suggests that along with the reisomerization also a re-oxidation occurs in the visual cycle. Regeneration of rhodopsin from added all-trans retinol has been achieved in homogenates of bleached eye tissues, including both retina and pigment epithelium (Hubbard and Wald, 1950 and 1951). Collins, Green and Morton (1953 and 1954) used a complex system, capable of maintaining oxidative phosphorylation, to study regeneration of rhodopsin. The capacity for regeneration of this system was determined and various components in the medium were then omitted, in order to find out the minimal requirements for regeneration. Their system contained retina, choroid and pigment epithelium. The complete medium contained cytochrome c, NAD, Mg^{2+}, ATP and a large excess of all-trans retinol. Bleached (78% complete) frog eye tissues incubated in the complete medium regenerated for 92% in one hour. Cytochrome c, NAD, ATP and retinol were all shown to be necessary. In the absence of these compounds, the regeneration fell to 45 to 55%. In the absence of oxygen regeneration fell to 71%. The amount of regeneration also decreased, when the tissue preparation aged. Rat rhodopsin regenerates 75% in 1 h in a medium containing only phosphate buffer, Mg^{2+} and a large amount of all-trans retinol and with the retina as the only tissue present. The fact that the presence of ATP was not necessary, does not mean that it plays no role in the process of regeneration, since the eye tissues themselves may be capable of synthesizing ATP. For the regeneration of cattle rhodopsin only phosphate buffer and retinol were necessary, yielding 48 to 100% regeneration in 2 - 3 h (Collins, Green and Morton, 1954). No faster regeneration was obtained when retinol or retinaldehyde were added, indicating that the oxidation of retinol is not rate

limiting in these experiments.

It is not clear, whether in these experiments isomerization occured, since a large excess of all-trans retinol was used. If only 1% of the added retinol would have been in the 11-cis configuration, the regeneration could be explaned entirely by recombination of the 11-cis isomer with opsin.

1.3.4 Short or long cycle?

When regeneration involves transport of the all-trans isomer to and isomerization in the pigment epithelium and return transport to the receptor cell we call this the "long cycle" of regeneration. There have been several reports of stimulation of rhodopsin regeneration by the pigment epithelium (Boll, 1877; Kühne, 1878 and 1879; Ewald and Kühne, 1878; Hosoya and Sasaki, 1938; Bliss, 1951[b]). The experiments of Dowling (1960) clearly indicate a migration of retinol from the retina to the pigment epithelium in the rat. Hubbard and Dowling (1962) showed that in the frog pigment epithelium slow isomerization of all-trans retinol esters occurs during dark adaptation. The experiments of Krinsky (1958) also provided evidence for an isomerizing enzyme in cattle pigment epithelium. These experiments do not provide definite proof for the existence of an isomerase in pigment epithelium, but they make it very likely.

If isomerization takes place within the receptor cell, we call this the "short cycle". Experiments on rhodopsin regeneration in isolated rat retinas (Cone and Brown, 1969; Goldstein, 1970) and on cone pigments in man (Rushton, 1958) indicate that in certain cases reisomerization certainly can occur without retinol leaving the retina. These experiments suggest the presence of an isomerizing enzyme able to convert all-trans retinol or all-trans retinaldehyde to the 11-cis isomers. There must be an active site on a retinal protein,

possibly rhodopsin itself, for the reisomerization reaction.

The regeneration of the rat 580 nm cone pigment also occurs in the isolated retina without involvement of the pigment epithelium (Goldstein, 1970). Moreover, the cone pigments have a considerably higher turnover than the rod pigment rhodopsin. Since the cones are concentrated in the fovea, there must either be a rapid flow of 11-cis retinaldehyde to the fovea, or the fovea itself must contain a rapid isomerization mechanism. Apparently, there exists an isomerizing system for cone pigments in the retina. This enzyme must catalyze the isomerization of all-trans to the 11-cis isomer and may be present in the immediate neighbourhood of the cones or in the cones themselves.

The rhodopsin regeneration rates at equilibrium also provide indications in favor of the short cycle. At an illumination level leading to 50% bleaching, the initial bleaching rate is 50% per minute (Rushton, 1957). The rate at 50% bleaching will be 25% per minute, since the bleaching rate is proportional to the rhodopsin concentration. This means that there is a complete turnover of pigment every 4 minutes. Long lasting illumination (15 min) does not influence the equilibrium level, so the amount of resynthesized rhodopsin in 15 min, must be 4 times the original amount of rhodopsin. Since the store of 11-cis retinol is probably not large enough for more than one replacement, fast isomerization must have occurred. If this isomerization reaction is a part of the "long cycle", the migration rate would have to be 10 times as high as in the rat (Dowling, 1960). This very fast migration seems rather unlikely, so probably a "short cycle" isomerization is of greater importance in the human eye.

In cephalopods the existence of the short cycle is even more obvious. These animals posses a system that isomerizes the all-trans retinaldehyde of the stable acid metarhodopsin

(comparable to the vertebrate metarhodopsin II, except that the latter is not stable) into the 11-cis isomer. Rhodopsin subsequently regenerates in the dark (Hubbard and StGeorge, 1958; Brown and Brown, 1958; Hamdorf, Schwemer and Täuber, 1968).

The linearity of the first part of the regeneration curve of the rat (Zewi, 1939; Lewis, 1957; Dowling, 1960 and 1963), frog (Weale, 1953; Reuter, 1964[a] and 1966), rabbit (Rushton, Campbell, Hagins ans Brindley, 1955) and cat (Rushton, 1953) can be explained as the result of two gradual changes, the first a decrease in metarhodopsin III concentration and the second an increase in rhodopsin concentration. Metarhodopsin III still has an appreciable absorption at 500 nm and is therefore easily confused with rhodopsin, when regeneration is measured as the increase in absorbance at 500 nm only and not by changes in the spectrum. More probable is the existence of a linear rate-limiting factor in the formation or availability of 11-cis retinaldehyde in the rod outer segments. Every minute the same amount of 11-cis retinaldehyde becomes available for reacting with opsin. The opsin concentration is so high and the reaction rate so fast, that every molecule of 11-cis retinaldehyde must be caught immediately by opsin to form rhodopsin. It is rather difficult to explain the nature of this linear rate-limiting factor.

In the rat eye the bleaching product all-trans retinol migrates to the pigment epithelium, when the eye is light-adapted. Upon dark adaptation, retinol returns to the retina, still in the all-trans configuration (Dowling, 1960). All-trans retinaldehyde or all-trans retinol are isomerized in the retina. In frog, where trans to 11-cis isomerization can occur in the pigment epithelium, the isomerization reaction is not rate limiting and might occur in the pigment epithelium as well as in the retina (Hubbard and Dowling, 1962).

A regulation at the level of the retinoldehydrogenase

by means of a limited oxygen supply is ruled out by Reuter (1966). A limitation by other chemical reactions of the rhodopsin cycle is rather improbable, since the linear regeneration phase of rat rhodopsin in vivo is nearly unaffected by a change in temperature of 10°C (Lewis, 1957). It seems more likely that some essential compound (e.g.retinol) must diffuse from the inner segment or elsewhere (e.g. pigment epithelium) to the outer segment. During the linear phase of the regeneration, the scarcity of this supply is the rate limiting factor.

The regeneration in animal eyes is linear until a point (about 75% regeneration) is reached where free opsin molecules become so few, that the rate of regeneration slows down and becomes proportional to the amount of free opsin. In the human eye and perhaps also in the rat eye (Tansley, 1931) the entire course of regeneration can be described by an exponential curve. The fast action of a retinal isomerase causes the level of 11-cis retinaldehyde to be constant. The regeneration is therefore only dependent on the opsin concentration and follows an exponential course.

It seems that there are two mechanisms of regeneration, one resulting in a linear course and another one in an exponential course. In animals like frog, rat and rabbit the linear mechanism is detected, in human only the exponential one. There is no solid evidence for the anatomical site of the isomerization reaction. In vertebrate retina there seem to be two systems capable of reisomerizing all-trans isomers to the 11-cis configuration, one operating in the retina (short cycle), and one in the pigment epithelium. When the latter system is used, the retinol must leave the retina during bleaching (long cycle). Not much is known about the chemical nature of the isomerization reaction. Retinyl ester, retinol or retinaldehyde or any combination of these, may serve as substrate. Neither do we know whether the isomerization takes place before or after recombination of the

retinaldehyde with opsin. There is no proof whether or not opsin is also involved in the energy utilizing reisomerization, but if reisomerization occurs in the retina, the active site of the isomerase is most probably located in the rod sac membrane.

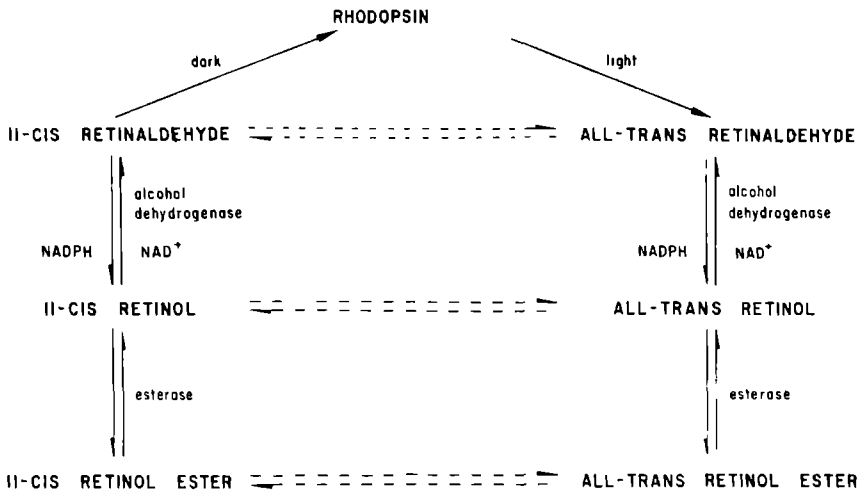


Fig.7 Reactions of the vertebrate rhodopsin cycle.
(dotted lines indicate speculative reactions)

1.4 RENEWAL OF ROD OUTER SEGMENTS

Indepently of the resynthesis of rhodopsin molecules from the products of its decomposition, there is a continuous renewal of rod outer segments, including the pigments contained therein. Radioautographic work has provided solid evidence of this renewal process. Protein synthesis occurs in the inner segment (Droz, 1963), from where the new proteins migrate rather rapidly to the base of the rod outer segment. Young (1967) and Young and Droz (1968) followed the incorporation of protein in rat, mouse and frog rod outer segments

for several weeks after administration of radioactive amino acids. They used electronmicroscopic autoradiography to detect the incorporated radioactive proteins. In frog, there was an incorporation of radioactive material at the basal end of the rods within 24 h. In the course of about 6 weeks the radioactive material moved in a well defined front along the axis of the rod. Finally the radioactive material reaches the apical site of the rods, where the rods are shed in groups of 8 - 30 and removed through phagocytic action of the overlying pigment epithelium (Young, 1967 and 1971; Young and Bok, 1969). The whole process takes about 6 weeks in the cold-blooded Rane pipiens and 10 days in the warm-blooded rat and mouse. The renewal of cones if occuring at all, is probably by another process, since radioactive material is found distributed diffusely throughout the outer segment (Young and Droz, 1968).

Bridges and Yoshikami (1969) have investigated the replacement of the retinaldehyde, needed for the renewal of the rhodopsin. After injecting rats with labelled retinaldehyde, they kept part of the animals in the dark and part of the animals were placed in the light and dark alternating every 12 hrs. Seven days after the injection the amount of radioactive chromophoric retinaldehyde was greatly increased. In the next 7 day period no further increase was observed. This time course corresponds with the rate of outer segment renewal in the rat (Young, 1967). Thus, the experiments of Bridges are probably another way to measure the renewal of rod outer segments. The light-dark history of the animals had no influence on the incorporation of radioactive retinaldehyde, so the rod renewal is independent of the resynthesis of rhodopsin after photolysis.

CHAPTER 2

THE NATURE OF THE CHROMOPHORIC GROUP OF RHODOPSIN

2.1 INTRODUCTION

Although it is generally accepted that 11-cis retinaldehyde is the chromophoric group of the visual pigment rhodopsin (Hubbard, Bownds and Yoshizawa, 1965; Bridges, 1967; Bonting, 1969, Morton and Pitt, 1969), critical examination of the original evidence for this opinin leads to the somewhat startling conclusion that direct proof for the presence of 11-cis retinaldehyde in rhodopsin has never been presented.

The original evidence is based on two observations. The first observation is that 11-cis retinaldehyde is the only retinaldehyde isomer that reacts with opsin to form rhodopsin (Hubbard and Wald, 1952; Wald and Brown, 1956). However, this does not prove that 11-cis retinaldehyde is present as such in rhodopsin. During this reaction the absorbance of 11-cis retinaldehyde at 360 nm disappears with the concomittant appearance of the rhodopsin peak at 500 nm, which is 1.7 times the height of the original 11-cis retinaldehyde absorbance peak. Since the other retinaldehyde isomers have all molar absorbances 1.7-1.8 times that of 11-cis retinaldehyde, this could mean that upon combination of 11-cis retinaldehyde with opsin the resulting chromophoric group would be another retinaldehyde isomer.

The second observation was made by Hubbard (1958), who obtained upon thermal denaturation of rhodopsin a product, which after incubation with opsin yielded rhodopsin. In a similar experiment with isorhodopsin, a rhodopsin analogue resulting from the reaction of opsin with 9-cis retinaldehyde, isorhodopsin was formed upon addition of

excess opsin. This is indeed suggestive for the conclusion that the chromophoric group is liberated in the same configuration that it had in the visual pigment, but the chromophoric groups were neither isolated, nor positively identified otherwise that by their reaction with opsin.

In view of the obvious importance for the understanding of the visual mechanism, we decided to seek direct and incontrovertible proof that 11-cis retinaldehyde is the chromophore of rhodopsin.

Another vexing point is the uncertainty concerning the molar absorbance of rhodopsin at the major absorbance peak at 500 nm. The original value of 40,600, determined by Wald and Brown (1953) has been challenged by Heller (1968), who reports a value of 23,100 for a purified rhodopsin preparation. The high value of Wald and Brown has been confirmed by Futterman and Saslaw (1961), Bridges (1970 and 1971), Shichi, Lewis, Irrevere and Stone (1969) and Daemen, Borggreven and Bonting (1970), who report values ranging between 40,900 and 43,000. These high values were all obtained by determining the 500 nm absorbance of a rhodopsin preparation and by chemical determination of its retinaldehyde content (as the oxim by Wald and Brown and by Bridges; with the thiobarbituric acid method by the other authors).

Heller (1968) determined, instead of the retinaldehyde content, the molar amount of rhodopsin by dividing the protein weight (determined by amino acid analysis) by the molecular weight (determined by calibrated gel filtration). He contended that the other approach suffered from low results in the chemical determination of retinaldehyde in rhodopsin. Although in an earlier study from our laboratory (Daemen, Borggreven and Bonting, 1970) we had shown reasonable evidence for the reliability of the method, a further report by Heller and Hall (1971) made us decide

to look for a method independent of the chemical determination of retinaldehyde. The experiments of Wald and Brown (1956) on the synthesis of rhodopsin suggested to us such a method, namely the comparison of absorbancies of rhodopsin and of an equivalent amount of its chromophore.

In this chapter we describe experiments involving the extraction and identification of the chromophore of rhodopsin, which prove directly that 11-cis retinaldehyde is indeed the chromophore. In addition we apply this approach to a determination of the molar absorbance of rhodopsin, which does not involve a chemical determination of retinaldehyde.

2.2 MATERIALS AND METHODS

2.2.1 Isolation, enrichment and determination of rhodopsin

All rhodopsin preparations were obtained from cattle eyes. All manipulations were carried out in darkness or in dim red light in a nitrogen atmosphere. Immediately after death, the eyes were excised and placed in a light-tight container at room temperature. Within two hours the retinas were dissected in the following manner. An incision was made at the ora serrata to remove the cornea. The lens and the vitreous body were then expelled by light pressure on the eye ball. Two incisions of about 1 cm were made at the edge of the eyecup. The retina was loosened cautiously and finally the connection with the optic nerve was cut. Retinas from 60 - 80 cattle eyes were homogenized in 30 ml ice-cold TRIS-HCl buffer (0.16 M, pH 7.1) by means of a loosely fitting Potter-Elvehjem homogenizer. During this homogenization the outer segments are detached from the rest of the retina. The homogenate was filtered through 120-mesh stainless steel wire screen under cautious stirring. The residue was washed with the same TRIS-HCl buffer and the combined filtrates

were mixed with aqueous sucrose to a final sucrose concentration of 0.42 M. From this suspension and an aqueous 1.28 M sucrose solution, continuous gradients with a density range of 1.05 to 1.18 were prepared. Centrifugation in a swing-out rotor (1 hr, 27,000 x g, 10°) yielded a heavy sediment and two layers near the middle of the tube. The upper layer contained the rod outer segments.

The rod outer segments thus obtained contained in most cases a certain amount of opsin. All of the opsin could be converted to rhodopsin by incubation with excess 11-cis retinaldehyde (25 nmol per retina). In order to obtain a very fine suspension of the retinaldehyde, the isomer was dissolved in 100 µl acetone. The isolated rod outer segments were added to this solution under vigorous stirring. The suspension was incubated for 1 hr under nitrogen at room temperature. Removal of the excess retinaldehyde was accomplished by adding NADPH in a five-fold molar excess, permitting the retinoldehydrogenase, present in the preparations, to reduce excess retinaldehyde to retinol, which was removed by a second gradient centrifugation.

After isolation of the enriched rod outer segments, two to three washings with TRIS-HCl buffer or with distilled water were performed (centrifugation at 45,000 x g, 4°, 30 min). In most cases the preparation was lyophilized and stored at -20° in the dark. Analysis of the final product showed that no opsin (determined by reaction with 11-cis retinaldehyde), no free retinaldehyde (determined by the thiobarbituric acid method; Futterman and Saslaw, 1961) and nearly no retinol (determined spectrally) was present. Furthermore the enriched preparations do not differ from the non-treated rhodopsin preparations with respect to regenerating capacity and stability towards NH_2OH and $NaBH_4$. The whole isolation and enrichment procedure has been described in detail by de Grip, Daemen and Bonting (1972).

In order to determine the molar concentration of a rhodopsin suspension, 40 µl of a 10% Triton-X-100 solution and 10 µl of a 1 M NH_2OH solution, both in 0.1 M phosphate buffer (pH 6.3), were added to 250 µl rhodopsin suspension in the same buffer. Extraction was accomplished by vigorous shaking of the mixture for about 1 min. After centrifugation at 18,000 x g for 5 min, the supernatant was transferred to a 1-cm light path quartz micro-cuvette. Spectra were measured before and after exhaustive illumination by a 75 W tungsten lamp at a distance of 15 cm for 10 min through ultraviolet and infrared filters (GG 435 and KG 1 filters,thickness 3 mm each, Scott-Jena, Mainz, Germany). From the difference in absorbance at 500 nm before and after illumination (ΔA_{500}) and the molar absorbance of rhodopsin in 1% Triton-X-100 solution (40,300; Daemen, de Grip and Jansen, 1972), the concentration of rhodopsin was calculated. The addition of Triton-X-100 was omitted when rhodopsin already was in a detergent solution (digitonin, CTAB or Emulphogene).

The ΔA_{500} obtained at a concentration of 1 mg lyophilyzed preparation per ml, is called the ΔA^1_{500} and varied between 0.250 and 0.300 .

2.2.2 Photochemical preparation and separation of retinaldehyde isomers

All-trans, 9-cis and 13-cis retinaldehyde were obtained from Eastman Kodak, Company, Rochester, New York. The 11-cis retinaldehyde required for our experiments was prepared by a modification of the procedure of Brown and Wald (1956), communicated to us by Dr. W. Sperling (Institut für Neurobiologie, Kernforschungsanlage,Jülich, Germany). First, 0.5 g all-trans retinaldehyde dissolved in 1.0 l ethanol was illuminated by three 100 W tungsten lamps at 15 cm distance. During the illumination nitrogen was bubbled through the solution, which kept it stirred and oxygen free. The temperature was maintained

between -20 and 20°C. The photoisomerization was followed by analyzing samples on small TLC plates. Illumination was stopped immediately when equilibrium was reached, since further illumination causes drastic decomposition. The ethanol was evaporated in darkness at 20° and the residue was taken up in 5 ml benzene/hexane (1:9 v/v). Separation of the mixture of retinaldehyde isomers was carried out on a column (50 cm long, 16 mm inner width) packed with aluminum oxide (containing 10% water) suspended in benzene/hexane (1:9 v/v) at an elution rate of 3 ml/min. At first breakdown products came off (λ_m 330 nm) followed by fractions containing retinaldehyde (3 ml). Spectral detection of the individual isomers is impossible, since all isomers absorb maximally in the same region. Detection by analyzing each fraction by thin-layer chromatography does not distinguish between 11-cis and 13-cis retinaldehyde. No other system is known, in which these isomers can be separated.

Fortunately, 11-cis retinaldehyde can be distinguished from other isomers, by means of its absorption maximum at 250-260 nm. The absorption of all-trans retinaldehyde in this region is low, it is somewhat higher in the 9-cis and the 13-cis isomers, and highest of all in the 11-cis isomer. 11-cis retinaldehyde has the lowest absorbance at 360 nm of all isomers, while all-trans retinaldehyde has the highest value. The 11-cis isomer, therefore, has the highest A_{250}/A_{360} ratio of all these retinaldehyde isomers. By measuring this ratio in each fraction it was possible to determine which fraction contained 11-cis retinaldehyde. Ten µl of each fraction was dried in a nitrogen-current, the residue was dissolved in 3 ml hexane and the A_{250}/A_{360} ratio was determined. Fractions with a value of 0.65 contain only 11-cis retinaldehyde. A lower A_{250}/A_{360} ratio indicates the presence of 13-cis retinaldehyde. Taking only the fractions with a A_{250}/A_{360} ratio of 0.65, 75 mg 11-cis retinaldehyde could be obtained from 0.5 g all-trans isomer.

The 11-cis retinaldehyde was dissolved in 50 ml hexane and stored at -20°C in the dark. All manipulations with samples containing 11-cis retinaldehyde were carried out under nitrogen and in red light to prevent oxidation and photo-isomerization.

2.2.3 Determination of retinaldehyde

The purity of the retinaldehyde isomers was checked by thin-layer chromatography, spectroscopic examination before and after iodine-catalyzed photoisomerization (Hubbard, 1956; Rotmans, Bonting and Daemen, 1972) and by reaction with opsin (see 3.2.5). Thin-layer chromatography was carried out in silica-gel with hexane/di-isobutylketone (11/2; v/v) or hexane/ether (85/15; v/v) as developing solvent and 9-cis, 11-cis and all-trans retinaldehyde as reference compounds. These two eluents gave similar resolution.

Iodine-catalyzed photo-isomerization of either 11-cis retinaldehyde or all-trans retinaldehyde leads to the same equilibrium mixture of retinaldehyde isomers with an apparent molar absorbance of 44,300 (Hubbard, 1956). The absorption spectrum of a retinaldehyde solution in hexane (1 ml, about 10^{-5}M) was measured, next 10 µl of a 50 µM iodine-solution in hexane was added both to measuring and blank cuvet, the cuvets were illuminated, 100 W tungsten lamp, at 15 cm from the cuvet, through an IR filter) until no further change in the absorption spectrum was observed. (about 30 sec) From the 360 nm absorbance of the extracted retinaldehyde in hexane before and after iodine-catalyzed photo-isomerization the composition of the original retinaldehyde mixture can be calculated by means of the known molar absorbances of pure 11-cis and all-trans retinaldehyde and of the equilibrium mixture after photo-isomerization.

This calculation is only possible, when no other than these two retinaldehyde isomers are present.

2.2.4 Extraction of retinaldehyde

The chromophoric group was isolated from rhodopsin by extraction with organic solvents, which spectrally denatured rhodopsin. We employed extraction with 90% ethanol or 90% acetone (1 ml/5 mg rhodopsin) for 10 min at 4° (Rotmans, Bonting and Daemen, 1972). The extract was centrifuged (5000 x g, 10 min, 4°) and the supernatant was diluted with 0.8 volume of ice-cold water. The diluted supernatant was extracted with an equal volume of hexane, which results in complete transfer of the retinaldehyde from the aqueous layer to the hexane layer. The percentage of retinaldehyde extracted from rhodopsin was calculated from determinations of retinaldehyde content in pellet, supernatant, and the original rhodopsin preparation by means of the thiobarbituric acid method of Futterman and Saslaw (1961) as modified by Daemen, Borggreven and Bonting (1970). The thiobarbituric acid reagent was prepared by dissolving 600 mg of thiobarbituric acid in 100 ml absolute ethanol. The solution was filtered and stored at 4°C. The thiourea reagent was prepared by dissolving 4 g thiourea in 100 ml of glacial acetic acid. The solution was filtered and stored at room temperature.

Samples were placed in Potter-Elvehjem tubes which could be centrifuged. After addition of 100 µl propanol and 1 ml of a freshly prepared mixture of thiobarbituric acid and thiourea reagents (1:1, by vol.) the mixture was homogenized, allowed to stand for 30 min, homogenized a second time and centrifuged for 10 min at 18,100 x g. The absorbance of the supernatant was measured at 530 nm.

Determination of the concentration and the stereochemical

composition of the extracted retinaldehyde could not be carried out satisfactorily on an extract of a normal rhodopsin preparation. This was apparently due to the presence of large amounts of (phospho)-lipids in the hexane-extract, since the problem was overcome by the use of delipidated rhodopsin. Therefore, we used in all experiments rhodopsin, which had been treated with phospholipase C and extracted with hexane.

2.2.5 Preparation of phospholipase C from Bacillus Cereus

Phospholipase C is obtained from cultures of B.Cereus. Ten ml of an overnight culture in brain-heart infusion are added to 1 l of a culture medium, containing 10 g pepton, 10 g yeast extract, 5 g NaCl and 0.4 g Na_2HPO_4 in water. The culture was incubated for 6 hrs at 37°C under aeration. The bacteria were sedimented by centrifugation for 20 min at 12,8000 x g. The remaining enzyme solution was concentrated by means of ultrafiltration through a diaflo membrane (type UM-10) mounted in an Amicon ultrafiltration cell. The concentrated solution was purified by Sephadex-G-100 column chromatography (Zwaal, Roelofsen, Comfurius and van Deenen, 1971; Otnaess, Prydz, Bjørklid and Berre, 1972).

2.2.6 Preparation of phospholipase C-treated rhodopsin

For the incubation with phospholipase C, fresh non-lyophilized rhodopsin preparations in 0.2 M Tris-maleate buffer (pH 7.2) were homogenized with an aliquot of the purified enzyme solution and shaken for 3 hrs at 37°C under nitrogen. After centrifugation for 10 min at 18,100 x g the sediment was washed twice with distilled water and was lyophilized. On this preparation a phosphorus determination was done (Borggreven, Rotmans, Bonting and

Daemen, 1971). Part of the phospholipase C treated preparation was washed with 0.067 M phosphate buffer pH 6.3, illuminated and incubated with 11-cis retinaldehyde.

2.3 RESULTS

2.3.1 Thin-layer chromatography of the extracted retinaldehyde

The chromophoric group was isolated from rhodopsin by extraction with the following solvents: 50% acetic acid, 50% dimethylformamide, 50-90% ethanol and 50-90% acetone. Complete extraction of the group required prolonged or repeated treatment at higher temperatures, but under these conditions thin-layer chromatography demonstrated considerable aspecific (light-independent) isomerization of the retinaldehyde isomers. Therefore, as a reasonable compromise we employed extraction with 90% ethanol for 10 min at 0^{o}, which yields 50-60% extraction of chromophore and less than 25% isomerization.

The thin-layer chromatogram of the hexane extract of the chromophore showed two Carr-Price positive spots, a major spot with the R_f of 11-cis retinaldehyde (which is also the R_f of 13-cis retinaldehyde) and a minor spot (about 20%) with the R_f of all-trans retinaldehyde. When the rhodopsin preparation had been illuminated before extraction, these ratios were reversed, the spot with the R_f of all-trans retinaldehyde now being the major spot (fig.8). Similar experiments with isorhodopsin (obtained by bacterial action on photolyzed rhodopsin; see chapter 6) showed 9-cis retinaldehyde to be the chromophoric group, whereas illuminated isorhodopsin yielded mainly all-trans retinaldehyde (fig.8).

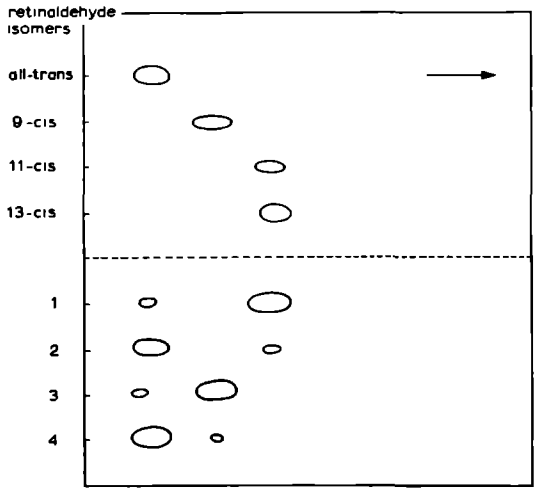


Fig.8 Thin-layer chromatography of chromophoric group extracted from visual pigments. Extraction by 90% ethanol at $0^{o}C$, of rhodopsin (1), illuminated rhodopsin (2), isorhodopsin (3) and illuminated isorhodopsin (4). Eluent: ether/hexane (15/85; v/v). Detection: spraying with Carr-Price reagent.

2.3.2 Reaction of extracted retinaldehyde with opsin

For the reaction with opsin the hexane extract of the chromophore was evaporated in a gentle stream of nitrogen, taken up in 1% buffered digitonin and mixed with an excess of opsin in digitonin solution and incubated for two hours in the dark (Brown and Wald, 1956). Opsin was prepared in the same way as rhodopsin, except that the isolation procedure was carried out in the light. Formation of rhodopsin was evident from a rise in absorbance at 500 nm, which completely disappeared after illumination in the

presence of 0.05 M hydroxylamine. After applying a correction for the incomplete extraction of the chromophoric group it was found that 60-75% of the amount of rhodopsin extracted could be regenerated upon incubation of the extracted chromophoric group with opsin. The part of the extracted chromophoric group, not reacting with opsin, could be fully accounted for the amount of all-trans isomer formed during the extraction (fig.8) while in addition always some isomerization of the 11-cis to the all-trans isomer occured during the incubation with opsin. The percentage of regeneration also excluded the presence of appreciable amounts of 13-cis retinaldehyde, which does not yield a photolyzable pigment with opsin.

2.3.3 Iodine-catalyzed isomerization of the extracted retinaldehyde

The identity of the major retinaldehyde compound in the chromophoric group extract as 11-cis retinaldehyde was definitely established by its absorption spectrum and the changes in this spectrum upon iodine-catalyzed photo-isomerization to a mixture of retinaldehyde isomers (Hubbard, 1956). All retinaldehyde isomers are converted by light to the same equilibrium mixture of isomers. Light, however, also destroys retinaldehyde. To hold destruction to a minimum, the stereo-isomerization was speeded up by adding a catalytic amount of iodine. On activation by light, the iodine molecule - or perhaps an iodine atom resulting from its dissociation - adds to a double band, converting it to a single bond capable of some degree of free rotation. When the iodine dissociates again, the double bond is reconstituted in either the cis or trans configuration. Iodine catalyzed photo-isomerization of the extracted retinaldehyde could not be carried out

satisfactorily on an extract of a normal rhodopsin preparation. This was apparently due to the presence of lipids in the hexane-extract, since the problem was overcome by the use of delipidated rhodopsin. Therefore, we used phospholipase C treated, hexane-extracted rhodopsin from which more than 95% of the phospholipids of the original rhodopsin preparation had been removed without affecting the spectral properties of the pigment. The absorption spectrum of the extract of the chromophore from this preparation showed a maximum at 363 nm (fig.9).

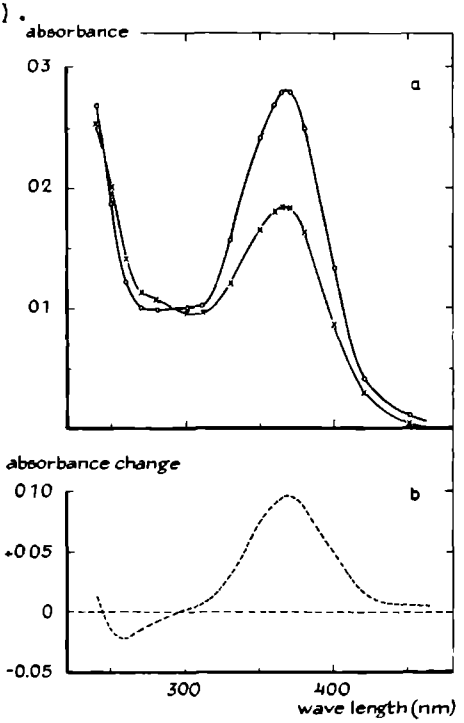


Fig.9 Iodine catalyzed photoisomerization of extracted retinaldehyde in hexane. (a) Absorption spectrum of chromophoric group extracted from lipid-free (phospholipase C treated, hexane extracted) rhodopsin before (x - x) and after (o - o) iodine-catalyzed photoisomerization. (b) Difference spectrum of curves depicted in (a).

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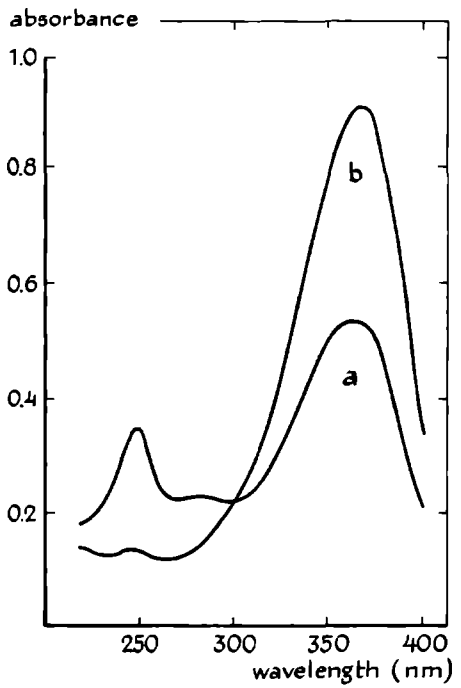

Fig 10.Iodine catalyzed photoisomerization of pure 11-cis retinaldehyde in hexane. Absorption spectrum of 11-cis retinaldehyde before (a) and after (b) iodine-catalyzed photoisomerization

Upon addition of a catalytic amount of iodine followed by illumination, the absorption maximum shifted about 3 nm to longer wavelenth , the absorbance near 365 nm rose by a factor 1.54, and the absorbance at 250 nm decreased slightly (fig.9, see also difference spectrum). The only retinaldehyde isomer that exhibits these changes in spectral characteristics during iodine-catalyzed photo-isomerization, is 11-cis retinaldehyde (fig.10) (Hubbard, 1956). Thin-layer chromatography indicated that after iodine-catalyzed photo-isomerization the original major spot corresponding to 11-cis retinaldehyde had changed to the spot corresponding to all-trans retinaldehyde.

From the absorbances of the extracted retinaldehydes in hexane before and after iodine-catalyzed photo-isomerization, the composition of the original retinaldehyde mixture can be calculated by means of the known molar absorbances of pure 11-cis and all-trans retinaldehyde (26,400 and 47,400, respectively; Kropf and Hubbard, 1970) and of the equilibrium mixture after photo-isomerization 44,300 ; Hubbard, 1956). When the extraction was performed at 4^{o}, the extracted retinaldehyde contained 76% (S.E.: 0.3, 4 expts.) 11-cis isomer. In the two experiments performed at 25^{o} only 35 and 50% 11-cis isomer was present in the extracted retinaldehyde, indicating a higher degree of non-specific isomerization at this higher temperature of extraction.

2.3.4 Calculation of the molar absorbance of rhodopsin

The molar absorbance of rhodopsin was calculated from the absorbance of the extracted retinaldehyde, while applying a correction for incomplete extraction and taking into account non-specific isomerization to all-trans retinaldehyde (Rotmans, van de Laar, Daemen and Bonting, 1972).

The percentage of retinaldehyde extracted from rhodopsin was estimated from determinations of retinaldehyde content in pellet and supernatant (see 2.2.4) by means of the thiobarbituric acid method (Futterman and Saslaw, 1961; Daemen, Borggreven and Bonting,1970). Parallel determinations on the original rhodopsin preparation yielded values equal to the sum of the values for pellet and supernatant determinations. It should be noted that the thiobarbituric acid method was used only to determine the percentage extraction of retinaldehyde from the ratios of retinaldehyde in pellet and supernatant, but not to determine the absolute amount of retinaldehyde. Partial non-specific isomerization of retinaldehyde has no effect on this determination, since all retinaldehyde isomers have nearly the same chromogenicity in the thiobarbituric acid method (Daemen, Borggreven and Bonting, 1970; Zorn and Futterman, 1971).

The calculation of the molar concentration of retinaldehyde by absorption spectroscopy in the hexane extract is complicated, because the original 11-cis isomer is partly isomerized to the all-trans isomer, and the molar absorbancies of 11-cis and all-trans retinaldehyde are very different (26,400 and 47,400 respectively; Kropf and Hubbard, 1970). This problem can be circumvented if the absorbance after iodine-catalyzed photoisomerization is measured. The iodine-catalyzed photoisomerization of either 11-cis or all-trans retinaldehyde leads to the same equilibrium mixture of retinaldehyde isomers with an apparent molar absorbance of 44,300 (Hubbard, 1956). Thus, the measurement of the absorbance of a retinaldehyde mixture after iodine-catalyzed photoisomerization permits the calculation of the total molar amount of retinaldehyde, irrespective of the composition of the original isomeric mixture. This method was applied to the retinaldehyde

extracted from rhodopsin.

Treatment with 90% ethanol or 90% acetone at 4°C yields extraction percentages from 43 to 69%, while at 25° 90 to 92% of the chromophore was extracted. After measurement of the absorbance of the extracted retinaldehyde after iodine-catalyzed photoisomerization, the total molar amount of retinaldehyde was calculated.

The molar absorbance of rhodopsin can be calculated in the following way. The amount of retinaldehyde really extracted equals the amount of chromophore on a molar basis, or expressed mathematically:

$$a \cdot \frac{A_{500}}{E_m} = \frac{A_{365}}{44,300}$$

where a is the fraction of retinaldehyde extracted, A_{500} the absorbance at 500 nm of a given concentration of a rhodopsin preparation in 1% triton-X-100, E_m the molar absorbance of rhodopsin, A_{365} the absorbance of the retinaldehyde extracted from this rhodopsin in an equal volume of hexane after iodine-catalyzed photoisomerization.

The results of six experiments are summarized in Table I. In the first four experiments the extraction was carried out at 4°, in the last two at 25°. In the latter case a higher extraction percentage was obtained, but the non-specific isomerization was also increased. We obtained a mean molar absorbance value of 43,250 (S.E.: 1010, 6 experiments).

Table I

The molar absorbance of cattle rhodopsin calculated from the spectral properties of extracted chromophore

Prepn. nr.		Extraction temp. (°C)	Chromophore extracted (a)	Molar absorbance calculated
1	90% ethanol	4	0.43	45,000
1	90% ethanol	4	0.54	41,000
2	90% ethanol	4	0.50	39,400
2	90% acetone	4	0.69	44,100
2	90% ethanol	25	0.90	44,300
3	90% ethanol	25	0.92	45,700
Mean value with standard error:				43,250 ± 1010

2.4 DISCUSSION

The essential step in the identification of the chromophore as well as in the determination of the molar absorbance, described in this chapter, is the extraction of the chromophoric group of rhodopsin in darkness by means of organic solvents. Thin-layer chromatography indicated that various solvents are able to extract retinaldehyde. When the extractions were performed at room temperature, almost only all-trans retinaldehyde was found. Extraction at 0°C with either 90% ethanol or 90% acetone, however, yielded another retinaldehyde

isomer. This indicated that rhodopsin contains a retinaldehyde isomer, which is less stable than all-trans retinaldehyde. The change of the spectral properties of the extracted retinaldehyde upon iodine-catalyzed photoisomerization showed that the major part of this retinaldehyde isomer has the 11-cis configuration. Unless 11-cis retinaldehyde is an artefact of the solvent extraction, which is very unlikely on energetic grounds (Kropf and Hubbard, 1970), these experiments directly prove the presence of 11-cis retinaldehyde as the chromophoric group of cattle rhodopsin.

A new method for the determination of the molar absorbance of rhodopsin was presented, in which this parameter is calculated from a comparison of the absorbance of rhodopsin in detergent solution with the absorbance of its extracted chromophore in hexane solution. A quantitative correction for incomplete extraction of the chromophore was applied, while the problem of non-specific isomerization of the chromophore was eliminated by determining the chromophore absorbance after iodine-catalyzed photoisomerization. Hence, this method did not require the use of pure rhodopsin, knowledge of the molecular weight of rhodopsin, or a chemical determination of retinaldehyde. The resulting molar absorbance value of 43,250 $\pm$ 1010 is close to the high value originally reported by Wald and Brown (1953) and confirmed by Bridges (1970 and 1971) Futterman and Saslaw (1961), Shichi, Lewis, Irrevere and Stone (1969) and Daemen, Borggreven and Bonting (1970). The discrepancy between the low molar absorbance value of 23,100 reported by Heller (1968 and 1970) and the high value around 41,000 obtained by other investigators is probably due to impurity of Heller's preparation and the use of an erroneously low molecular weight of 26,400. The impurity is very likely due to the presence of opsin, which constitutes about 10 - 20% of the weight in nearly

all rhodopsin preparations. More recently, Daemen, de Grip and Jansen (1972) in our laboratory have calculated the molar absorbance in exactly the same way as Heller did, but using preparations containing no opsin and using a more correct value for the molecular weight. They obtained a value of 39,950 $\pm$ 1650, which again confirms the high value of Wald and Brown (1953). Since our method did not depend on the determination of retinaldehyde criticized by Heller (1968) and because Daemen, de Grip and Jansen(1972) employed the same method as used by Heller (1968), we can now be sure that Heller's low value is not correct.

CHAPTER 3

REACTION OF 11-CIS RETINALDEHYDE WITH OPSIN

3.1 INTRODUCTION

In the preceding chapter we have shown that 11-cis retinaldehyde is indeed the chromophore of cattle rhodopsin. When the rhodopsin molecule absorbs a photon of suitable wavelength, the chromophore is isomerized to all-trans retinaldehyde. This occurs in the first step of photolysis: rhodopsin $\longrightarrow$ prelumirhodopsin. The subsequent steps in the photolysis of rhodopsin do not require light and are thermal reactions, leading consecutively to lumirhodopsin, metarhodopsin I and metarhodopsin II. In all of these photolytic intermediates the all-trans retinaldehyde is still bound by means of an aldimine link to opsin. Since metarhodopsin II is formed within milliseconds, while its decay takes several minutes, the conversion of metarhodopsin I to metarhodopsin II is the last step which could trigger excitation. There are some arguments to believe that it actually is the crucial step (Bonting, 1969, pp 372-374). Hence it is important to know more about the fate of the chromophore during this conversion.

The chromophore binding site in metarhodopsin II has been shown to be the ε-amino group of a lysine residue in opsin (section 1.2.7). More recently evidence has accumulated to show that this is also true for rhodopsin (section 1.2.7). Since there are 16 lysine groups per molecule of opsin, the possibility of a transiminization from one lysine group to another during the metarhodopsin I to metarhodopsin II conversion exists. There were actually some arguments to assume the occurrence of such a transiminization (Bonting, 1969, p 374).

It occurred to us that this could be investigated by fixing the retinaldehyde to its binding site by treatment with $NaBH_4$, and then probing the original binding site by treatment with 11-cis retinaldehyde. Should this site have been vacated by the original chromophore, then formation of a new photopigment would be expected to occur.

Since the reaction between opsin and 11-cis retinaldehyde is a very crucial aspect of such a study, and since no previous studies of this reaction have been made with suspensions of rod outer segment membranes, it was necessary first to carry out such an investigation. The reverse of this reaction, the hydrolysis of retinylidene-opsin to opsin and retinaldehyde, has also been studied by us, since we wanted to be able to distinguish between vacation of the chromophore binding site by transiminization and by hydrolysis. The results of these two studies are reported in this chapter, while the results of the transiminization experiments will be set forth in the next chapter.

3.2 MATERIALS AND METHODS

3.2.1 Photolyzed rhodopsin

Rhodopsin is prepared as described in 2.2.1. This procedure gives preparations of high purity, containing no free opsin, no free retinaldehyde and virtually no free retinol. Photolyzed rhodopsin was prepared from a suspension of rhodopsin in 0.067 M phosphate buffer (pH 6.3) by illumination with a 75 W tungsten lamp for 10 min at a distance of 15 cm. For optimal bleaching we used an orange filter, which transmits only wavelengths longer than 550 nm, and an infrared filter to avoid thermal decomposition (OG 370 and KG 1 filters, thickness 3 mm each, Schott-Jena, Mainz, Germany). Orange light is used,

because it is not absorbed by the resulting all-trans retinaldehyde and hence does not isomerize this substance. Illumination with white light, which is capable of isomerizing retinaldehyde, bleaches only about 75% of the rhodopsin, orange light gives 90-95% photolysis. The remaining 5-10% rhodopsin is converted through photo-isomerization to a 1:1 mixture of rhodopsin and isorhodopsin.

3.2.2 Opsin

In photolyzed rhodopsin part of the retinaldehyde is still attached to the opsin by aldimine bonds. Opsin can be obtained by removal of this retinaldehyde. The aldehyde trapping reagent hydroxylamine is not very suitable since the resulting oxime cannot be washed out completely. Repeated extraction with hexane, even though it does not harm the opsin, is not suitable because it removes the retinaldehyde only partially. The best way is to reduce the retinaldehyde by means of the retinol-dehydrogenase activity present in the rod outer segment membranes. A five-fold molar excess of NADPH, which serves as coenzyme, was added to a 25 µM suspension of photo-lyzed rhodopsin. Upon incubation for 30 min at room temperature reduction to retinol was nearly complete, as was shown by a thiobarbituric acid determination of the remaining retinaldehyde. The resulting retinol could be removed by several washings with serum. A retinalde-hyde determination showed that less than 5% of the original amount of retinaldehyde was still present. Spectra of photolyzed rhodopsin, NADPH-treated photolyzed rhodopsin and opsin are shown together with the spectrum of the original rhodopsin in fig 11.

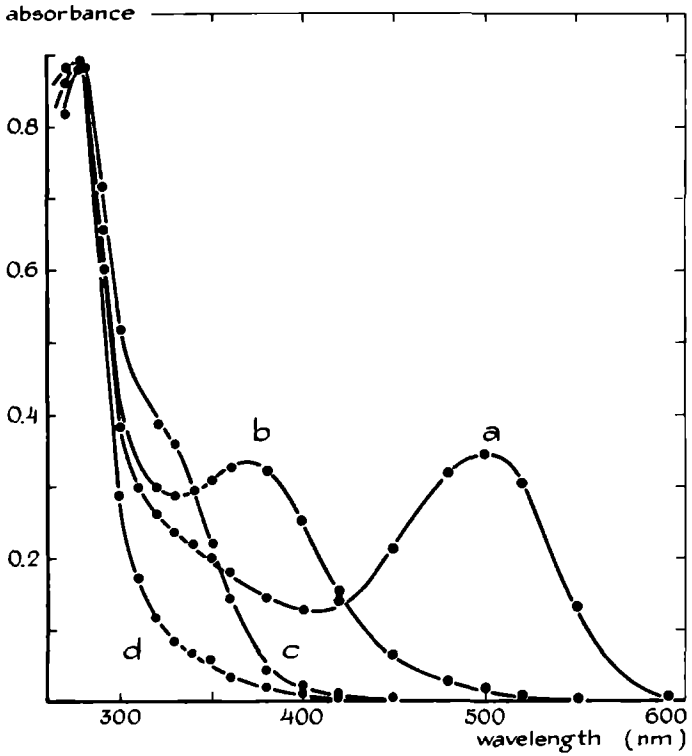

Fig 11. Spectra of rhodopsin (a), photolyzed rhodopsin (b), NADPH-treated photolyzed rhodopsin before (c) and after (d) extraction of retinol by human serum. Spectra were measured after solubilization of 1 mg lyophilized preparation in 1 ml 1% Emulphogene.

3.2.3 Reduction of retinylidene-opsin

When photolyzed rhodopsin is treated with $NaBH_4$, the aldimine bond is reduced to an amine link. This makes the link of the chromophore to opsin stable against hydrolysis.

To 1 ml of a 25 µM rhodopsin suspension in 0.25 M phosphate buffer (pH 6.7) 2 mg dry $NaBH_4$ (Fluka, Switzerland) was added. After vigorously mixing for a few seconds, the mixture was illuminated by a 100 W tungsten lamp (distance 15 cm) through ultraviolet and infrared filters. In some experiments the rhodopsin suspension was illuminated first and the $NaBH_4$ was added at various times thereafter. Part of the $NaBH_4$ reacted with water, which caused a small increase in pH (maximally about 0.3 pH). After illumination for 5 min the sample was centrifuged (35,000 x g; 15 min) to separate the resulting retinylopsin from the remaining $NaBH_4$. This procedure caused no loss of material. The pellet was resuspended in 0.25 M phosphate buffer (pH 6.7). After centrifugation the pellet was taken up in a small volume of 0.1 M phosphate buffer (pH 6.3) for further use. Spectra of illuminated rhodopsin, before and after reduction, were measured.

3.2.4 Protonation of retinylidene-opsin

Another way to fix the chromophore to the opsin molecule is by protonation of the aldimine bond. To 1 ml of a photolyzed rhodopsin suspension in 0.067 M phosphate buffer (pH 6.7), 100 µl 1 M HCl was added while the suspension is shaken. The protonated photolyzed rhodopsin was analyzed by comparing the absorbance of the protonated aldimine (λ_{max} = 440 nm) with that of the free retinaldehyde (λ_{max} = 380 nm). The concentrations of these compounds were calculated from these absorbancies by applying the following equations:

$$A_{440} = E_{m\ ret\ 440} \cdot C_{ret} \cdot D + E_{m\ ald^+ 440} \cdot C_{ald^+} \cdot D$$

$$A_{380} = E_{m\ ret\ 380} \cdot C_{ret} \cdot D + E_{m\ ald^+ 380} \cdot C_{ald^+} \cdot D$$

A_{440} and A_{380} are the measured absorbancies at these two wavelengths; $E_{m\ ret\ 440}$ and $E_{m\ ret\ 380}$ are the molar absorbancies of retinaldehyde and $E_{m\ ald^+\ 440}$ and $E_{m\ ald^+\ 380}$ the molar absorbancies of the protonated aldimine at 440 and 380 nm (de Pont, Daemen and Bonting, 1970[b]). D is the light path (in cm) of the cuvette and C_{ret} and C_{ald^+} are the concentrations of free retinaldehyde and its protonated aldimine. A simplified calculation of the latter concentrations is possibly by substituting for C_{ret} the expression $1-C_{ald^+}$ and dividing the two equations. The resulting expression,after insertion of the known molar absorbancies, can be plotted as percent protonated aldimine against the ratio A_{440}/A_{380}.

3.2.5 Reaction of opsin and 11-cis retinaldehyde

The preparation and determination of 11-cis retinaldehyde has been described in paragraph 2.2.2. The resynthesis of rhodopsin from opsin and 11-cis retinaldehyde was always carried out in darkness and under a nitrogen atmosphere. When no detergent was used, the 11-cis retinaldehyde needed for the recombination reaction was first dissolved in acetone. Under vigorous shaking 125 nmol 11-cis retinaldehyde in 50 µl acetone was added to 1 ml of a 25 µM suspension of opsin or retinylopsin in 0.067 M phosphate buffer (pH 6.3). In this way a fine suspension of the water insoluble retinaldehyde was obtained. There is no denaturation of the opsin, when the volume of acetone in which the 11-cis retinaldehyde is dissolved does not exceed 50 µl per ml suspension. When the incubation was carried out in detergent (digitonin, Triton-X-100, CTAB or Emulphogene), both opsin and 11-cis retinaldehyde were solubilized separately and mixed at the start of the incubation.

Experiments on the maximal regeneration obtained after

incubation with 11-cis retinaldehyde were performed at 37°C, while effects on the reaction rate were studied at 25°C. The amount of rhodopsin formed during regeneration can be estimated from the absorbance at 500 nm, the absorption maximum of rhodopsin. Such measurements are not entirely reliable, since retinaldehyde will also combine with other amino groups on the opsin molecule. The resulting compounds may show measurable absorption at 500 nm, especially in acid media. Moreover the all-trans retinaldehyde bound in these compounds is more sensitive to photoisomerization than free retinaldehyde. In media suitable for regeneration 11-cis and 9-cis isomers, resulting from photoisomerization during bleaching, may react with opsin giving rhodopsin and isorhodopsin, both absorbing at 500 nm. For these reasons the rhodopsin has always been measured after addition of hydroxylamine, which converts free as well as bound retinaldehyde to the oxime (λ_{max} = 360 nm) and leaves the rhodopsin unaltered.

For the measurement of the 500 nm absorbance, Triton-X-100 in a final concentration of 1% was added to an aliquot of the suspension, while the final hydroxylamine concentration was 0.05 M. The amount of regenerated rhodopsin was determined from the decrease in absorbance at 500 nm upon illumination and is expressed in percent of the amount of rhodopsin originally present in the preparation. This percentage is designated by us as the regeneration capacity.

3.3 RESULTS

3.3.1 Maximal regeneration

The stability of rhodopsin is mostly measured by two criteria, the maintainance of the 500 nm absorbance and the capacity to react with 11-cis retinaldehyde after

exposure to light. As far as the spectral properties are concerned, the rhodopsin preparations are quite stable. At room temperature between pH 4 and 10, no change was observed for at least 1 month.

When a maximally bleached rhodopsin (90 to 95% photolysis) was incubated with a five fold molar amount of 11-cis retinaldehyde for 3 h at 37°, the amount of resynthesized rhodopsin varied considerably with the pH. This is due to denaturation in acid and alkaline medium.

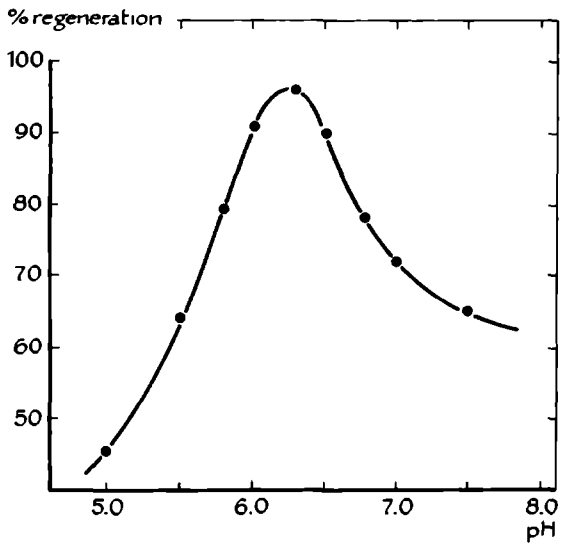

Fig 12. Rhodopsin regeneration as a function of pH. Photolyzed rhodopsin (5 nmol), solubilized in 1% digitonin dissolved in 0.067 M phosphate buffer, was incubated for 3 h with a 20-fold molar amount of 11-cis retinaldehyde. The amount of resynthesized rhodopsin was determined from ΔA_{500} and expressed as percentage of the rhodopsin originally present.

At pH 6.3 the reaction of photolyzed rhodopsin with 11-cis retinaldehyde is optimal (fig.12). The optimal temperature range for the recombination reaction is 30°-40° (fig.13), while at 0° no reaction occurs.

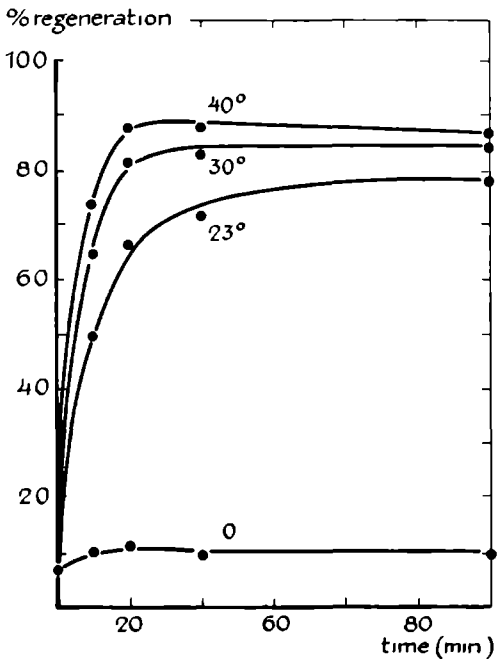

Fig 13. Synthesis of rhodopsin from photolyzed rhodopsin and 11-cis retinaldehyde at various temperatures. Rhodopsin (25 nmol) suspended in 1 ml 0.067 M phosphate buffer (pH 6.3) was illuminated for 5 min at room temperature through orange and infrared filters. After preincubation of the photolyzed rhodopsin 75 nmol 11-cis retinaldehyde was added. The amount of rhodopsin generated at various temperatures is expressed as percentage of rhodopsin originally present.

The resulting rhodopsin is relatively stable at temperatures between 30^{o} and 40^{o}.

At optimal pH and temperature, maximal regeneration varies from 50 to 100%, with an average of about 85% (10 experiments). Hence, on the average 15% of the photolyzed rhodopsin is denatured (i.e. rendered incapable of regeneration) during photolysis or subsequent incubation at 37^{o}. After renewed photolysis of the resulting preparation an average of 75% of the original rhodopsin is regenerated upon incubation with 11-cis retinaldehyde. This means that an additional 10% of the photolyzed rhodopsin is denatured when regeneration is carried out a second time.

The rate at which the regeneration capacity is lost upon aging, was different in each preparation and proceeded much faster than the loss of the 500 nm absorbance. In other words, the denaturation of opsin is much faster than that of rhodopsin, and the maintainance of the 500 nm absorbance of rhodopsin is clearly no good measure of the regeneration capacity of the rhodopsin complex. The observed variability makes it rather difficult to give exact data on the long-term denaturation of rhodopsin or photolyzed rhodopsin.

The regeneration capacity of rhodopsin solubilized in 1% digitonin and of the same rhodopsin suspended in 0.067 M phosphate buffer is exactly equal. Hence, the structure of the chromophore binding site is apparently not affected by the rather mild action of digitonin, which causes only partial fragmentation of the rod outer segment membrane. The insensitivity of the chromophore binding site to certain alterations in the membrane structure is also demonstrated by the fact that lyophilization, alum-treatment and hexane extraction of the rhodopsin preparation have no effect on the regeneration capacity. More effective detergents than digitonin such as Triton-X-100, CTAB and Emulphogene

cause complete loss of the regeneration capacity.

3.3.2 Effect of reactant ratio

The effect of the ratio of 11-cis retinaldehyde to opsin on the recombination reaction has also been studied. For this purpose we incubated different amounts of 11-cis retinaldehyde with excess opsin. After 3 h incubation, the increase in 500 nm absorbance stopped and maximal regeneration was achieved. In digitonin as well as in 40% sucrose, 50% glycerol and 0.067 M phosphate buffer (pH 6.3), the amount of rhodopsin formed in 3 hours was proportional to the amount of added 11-cis retinaldehyde, up to 80% regeneration. In hexane regeneration also occurred, but there was no linearity between the amounts of regenerated rhodopsin and of added 11-cis retinaldehyde. In suspension about 2 moles of 11-cis retinaldehyde are needed for the resynthesis of one mole of rhodopsin (fig 14). Over a number of experiments that ratio varied between 1.1 and 3.2, but a 1:1 ratio was never observed. This means that in the presence of excess opsin only 31 to 91% of the added 11-cis retinaldehyde was used for rhodopsin resynthesis.

The efficiency with which 11-cis retinaldehyde is used for rhodopsin resynthesis was virtually the same in 1% digitonin, 40% sucrose, 50% glycerol and 0.067 M phosphate buffer and depended only on the opsin preparation used. Also the prior removal of all-trans retinaldehyde from photolyzed rhodopsin had no effect on the recombination efficiency.

Spectra of regenerated rhodopsin samples show that upon addition of 11-cis retinaldehyde to an excess of opsin not only the absorbance at 500 nm but also the absorbance around 360 nm was raised (fig.15).

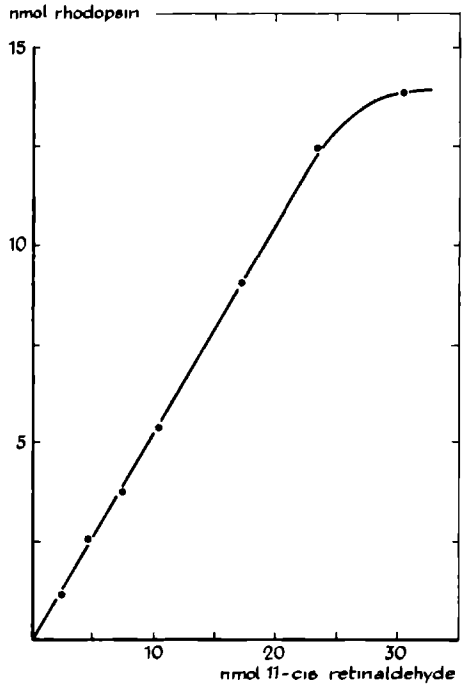

Fig 14. Synthesis of rhodopsin in phosphate buffer. Rhodopsin (14.5 nmol) was suspended in 1 ml 0.067 M phosphate buffer (pH 6.3). After addition of a 5-fold molar amount of NADPH, the sample was illuminated for 10 min through ultraviolet and infrared filters. After 45 min at 25°, the opsin was sedimented by centrifugation (20,000 x g, 15 min) and washed once with the same buffer. Then varying amounts (2.5 to 30 nmol) 11-cis retinaldehyde in hexane were pipetted into test tubes. After evaporation of the hexane by a stream of nitrogen, 20 µl acetone was added to redissolve the retinaldehyde. Under vigorous stirring 1 ml of the washed suspension was added and incubated for 3 h. The amount of rhodopsin resynthesized was calculated from ΔA_{500}.

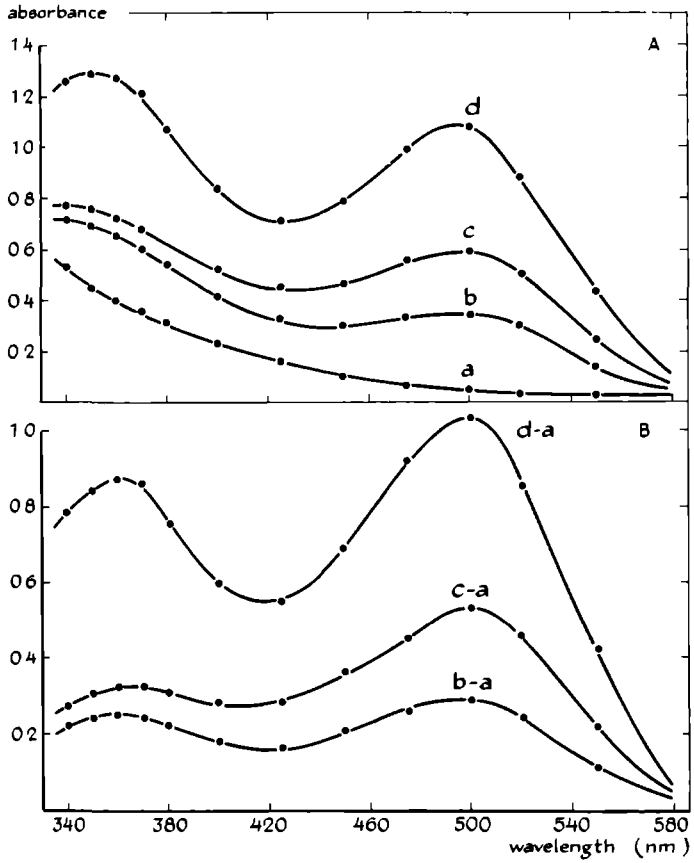

Fig 15. Spectra of regenerated rhodopsin. Opsin (30 nmol) was suspended in 1 ml 0.067 M phosphate buffer (pH 6.3). The spectrum (a) of this suspension was measured after solubilization in 1% Triton-X-100. After 3 h incubation with 15 nmol 11-cis retinaldehyde, a thousand-fold excess NH_2OH was added and the spectrum was measured after solubilization (b). Incubation with 30 and 60 nmol 11-cis retinaldehyde gave spectra c and d, respectively. Difference spectra are depicted in fig B.

This also indicates that not all added retinaldehyde was used for rhodopsin synthesis. In order to determine the fate of the remaining retinaldehyde, we removed, after completed regeneration, the non-chromophoric retinaldehyde by extraction with hexane. This extractant does not remove chromophoric retinaldehyde. Analysis of the extract by thin-layer chromatography (silica; ether/hexane, 15/85, v/v) showed that during incubation part of the 11-cis retinaldehyde had been isomerized to the all-trans isomer and was therefore no longer capable of reacting with opsin. In the absence of opsin the 11-cis isomer was not isomerized, since a solution of the isomer in digitonin incubated in the same way as for the regeneration experiments but in the absence of opsin, yielded no all-trans isomer. The isomerization of 11-cis retinaldehyde was rather non-specific, since it occurred with native rhodopsin, heated opsin, retinal proteins and even with bovine serum albumin. Moreover, 11-cis retinol was also isomerized upon incubation in the presence of opsin. In further experiments we always used a four-fold molar excess of 11-cis retinaldehyde for the determination of the maximal regeneration, since this excess is sufficient to overcome the non-specific isomerization and thus ensure maximal regeneration.

3.3.3 Effect of all-trans retinaldehyde

Since in photolyzed rhodopsin all-trans retinaldehyde, either in the free or bound form is present, we studied the possible effect of its presence on the reaction of added 11-cis retinaldehyde with the chromophore binding site. For this purpose we incubated freshly prepared photolyzed rhodopsin and retinaldehyde-free opsin with 11-cis retinaldehyde. No difference in reaction rate was observed. In addition, we preincubated photolyzed

rhodopsin with excess exogenous all-trans retinaldehyde prior to incubation with the 11-cis isomer. No effect on the rate of the reaction with 11-cis retinaldehyde was noticed, when up to an 8-fold molar amount of all-trans retinaldehyde was added (fig 16).

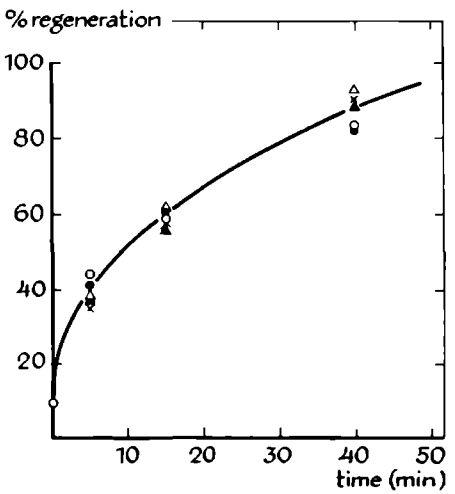

Fig 16. Lack of competition between 11-cis and all-trans retinaldehyde for the chromophoric site. Rhodopsin (30 nmol), suspended in 1 ml 0.067 M phosphate buffer (pH 6.3) was illuminated for 5 min through orange and infrared filters. In the dark 90 nmol 11-cis retinaldehyde was added. After 3 h incubation at 25° the amount of regenerated rhodopsin was measured. In parallel experiments various amounts of all-trans retinaldehyde (0 nmol o, 60 nmol ●, 150 nmol x, 270 nmol Δ, 720 nmol ▲) were added 5 min prior to the addition of the 11-cis isomer.

Thus, all-trans retinaldehyde has either a much lower affinity for the chromophore binding site than the 11-cis isomer, or it is not bound at all at this site.

3.3.4 Hydrolysis of retinylidene-opsin

This process has been studied by "fixing" the bound chromophore at various times after illumination, either by protonation or reduction, and determining the ratio of free to bound chromophore. Acidification of retinylidene-opsin yields a protonated aldimine (λ_{max}=440 nm), which is stable, while the spectrum of free retinaldehyde (λ_{max}= 380 nm) remains unaltered (figs 17 and 18).

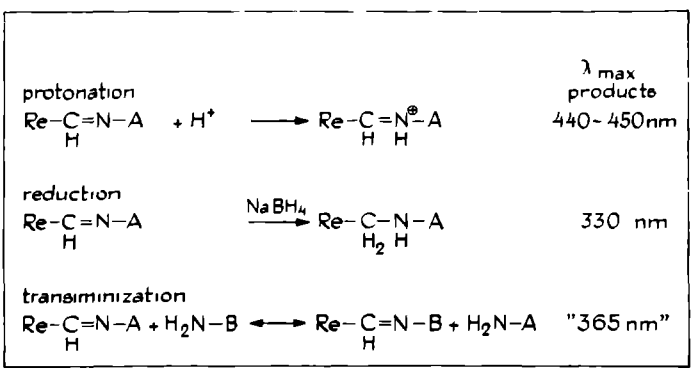

Fig 17. Reactions of retinylidene-imines

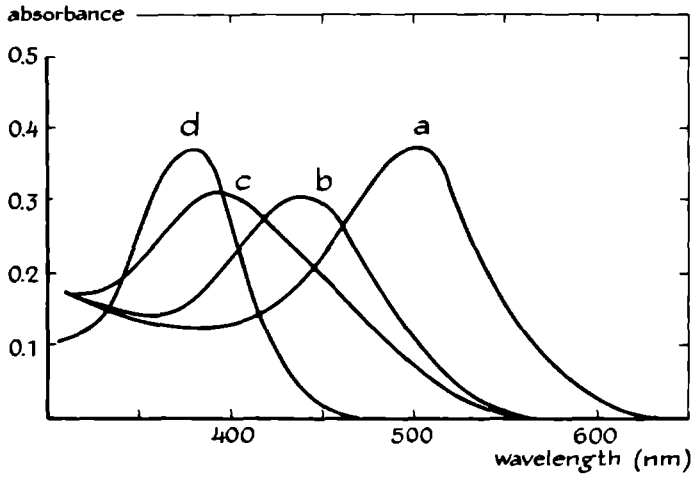

Fig 18. Hydrolysis of retinylidene-opsin, determined by the protonation method. Native rhodopsin suspended in 1 ml 0.067 M phosphate buffer at pH 6.3 (a) was protonated by addition of 100 µl HCl before (b) and 10 min after (c) illumination ended. Curve (d) represents the spectrum of 11-cis retinaldehyde in Triton-X-100 solution. All spectra were recorded after solubilization in 1% Triton-X-100.

A shift in the equilibrium between free and bound retinaldehyde through binding of free retinaldehyde is prevented, since the free amino groups on the opsin molecule also become protonated. From the ratio A_{440}/A_{380} the amount of free and bound retinaldehyde can be calculated (section 3.2.4). The second method is by reduction with $NaBH_4$, which converts 95 to 100% of the retinylidene-opsin to the stable retinylopsin (section 3.2.3; fig 17). In this case free retinaldehyde is reduced to retinol,which after treatment of the preparation by methanol can be extracted with hexane. In order to

examine the possibility that the reduced compound might be a retinylphospholipid, the hexane extract of denatured retinylopsin has been analyzed by thin-layer chromatography. The retinylopsin suspension was diluted with one volume of methanol and extracted four times with hexane. The hexane extract was subjected to thin-layer chromatography (silica, eluent: ether/hexane, 1/1, v/v). Reference substances were all-trans retinaldehyde, all-trans retinol and retinyl-phosphatidylethanolamine. After development the spots were detected by fluorescence and by spraying with Carr-Price reagent. It was shown that the hexane extract contained only a trace amount of retinylphospholipids. Thus, the absorbance of the hexane extract at 330 nm was mainly due to retinol and can be taken as a measure for the amount of free retinal-dehyde present at the moment of reduction. Both methods yielded the same results (fig 19).

It was found that a minimal and constant binding ratio was reached after 30 min, at which time only about 40% of the retinaldehyde had been released. The remaining 60% of the retinaldehyde was still bound the an amino group on the opsin molecule. It is very likely that this is mainly bound to the ε-amino group of a lysine residue. Only a minor part of the retinaldehyde is bound to amino group containing phosphoslipids. Thin-layer chromato-graphy of the hexane extract showed that retinylphospho-lipids first appear 30 min after illumination and gradually increase thereafter. However, the amount of retinylphospholipids always remained relatively small, less than 10% of the amount of retinol, as judged by the intensity of the spots on the thin-layer chromatogram. Thus, after 30 min 40% of the retinaldehyde is released, close to 60% is bound to opsin and less than 4% to phospholipids.

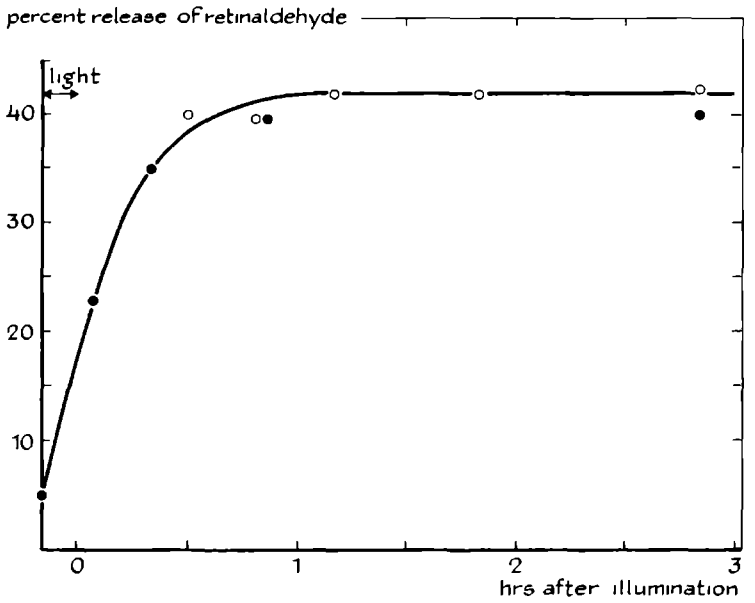

Fig 19. Retinaldehyde release from rhodopsin after illumination as a function of the time elapsing after illumination.
(○ determined by the protonation method,
● determined by $NaBH_4$ reduction).

3.4 DISCUSSION

It is a well known fact that rhodopsin retains its capacity to react with 11-cis retinaldehyde after photolysis better than opsin does. Apparently, conformational changes induced in the opsin structure upon release of 11-cis retinaldehyde make the molecule less stable. This phenomenon is analogous to the often observed increased stability of an enzyme in the presence of its substrate due to the "induced fitting" of the active site.

The main purpose of the studies reported in this chapter was to investigate this chromophoric site: the effect of detergents, the affinity for 11-cis and all-trans retinaldehyde and its state after photolysis.

In studies in which the recombination reaction is used to investigate the chromophoric site, it should be kept in mind that the in vivo process of rhodopsin resynthesis might differ considerably from the recombination reaction observed in vitro. For example, in previous studies of the recombination reaction (Wald and Brown, 1951; Hubbard and Wald, 1952[a] and 1952[b]; Hubbard. Gregerman and Wald, 1953; Wald, Brown and Kennedy, 1956; Hubbard, 1958) both the retinaldehyde and the illuminated rhodopsin were solubilized in digitonin solution because of their insolubility in water. Digitonin is, as far as we know, the only detergent able to dissolve rhodopsin without affecting its regeneration capacity. However, this detergent causes structural degeneration of the rod outer segment membrane, which hinders proper studies on the micro-environment of the rhodopsin molecule. Since we wanted to investigate hydrolysis and transiminization of the all-trans retinaldehyde after photolysis of rhodopsin, it was essential to preserve the original structure of the rod outer segment membrane. Therefore these experiments were performed with suspensions of disrupted rod outer segments. Under these conditions in vitro studies may give valuable information as to which groups or sites on the opsin molecule are involved in the reactions after photolysis.

First, we had to reinvestigate pH-optimum, temperature optimum, maximal regeneration and reactant ratio of the recombination reaction, since these data had only been reported for rhodopsin resynthesis in digitonin. Our studies showed that solubilization of rhodopsin was not required for resynthesis to occur. Our findings for the

pH dependence of rhodopsin regeneration in suspension and of the variable loss in regeneration capacity upon aging agree very well with the corresponding data of Radding and Wald (1956[a] and 1956[b]) on rhodopsin in digitonin solution.

Simultaneously with rhodopsin resynthesis from opsin and 11-cis retinaldehyde, there occurs a non-specific isomerization of the added 11-cis retinaldehyde to the all-trans isomer. As a result only 31 to 91% of the added 11-cis retinaldehyde was used for rhodopsin resynthesis. The observed variability in regeneration efficiency agrees with results of previously published studies with digitonin-solubilized rhodopsin (Hubbard and Wald, 1952; Hubbard, Gregerman and Wald, 1953; Brown and Wald, 1956). It seems that the relative rates of the recombination and isomerization reactions depend on the isolation procedure and the dispersion, and hence differ for each rhodopsin preparation. Alterations in the structure of the rod outer segment membranes may lead to a loss of optimal conditions for rhodopsin resynthesis.

The fact that the rate of recombination of opsin and 11-cis retinaldehyde is not decreased by high concentrations of all-trans retinaldehyde, indicates that the chromophore binding site has a high affinity only for the 11-cis isomer. This result is in contradiction with the observation of Dowling and Hubbard (1963) that the rate of rhodopsin resynthesis in the rat is increased when the incubation of photolyzed rhodopsin with 11-cis retinaldehyde is delayed for 30 min after illumination. We could not find such a difference in initial reaction rate under our experimental conditions. The suggestion that the initially lower rate of the recombination reaction could be due to the occupation of the chromophoric site by the all-trans retinaldehyde molecule during the first 30 min after

photolysis, is not tenable, since it was shown that all-trans retinaldehyde is not an inhibitor of the recombination reaction.

The low affinity of all-trans retinaldehyde for the chromophoric site does not necessarily signify a complete hydrolysis of retinylidene-opsin. On the contrary, we were able to establish that retinylidene-opsin in suspension hydrolyzes only partly to an equilibrium state, in which nearly 60% of the all-trans retinaldehyde is bound to amino groups of the opsin molecule and less than 4% to phospholipids. These opsin amino groups are probably ε-amino groups of lysine residues, just as in metarhodopsin (Bownds, 1967; Akhtar, Blosse and Dewhurst, 1967 and 1968). The methods employed by us (protonation and reduction) give the amount of covalently bound retinaldehyde. It seems rather likely that even retinaldehyde, which is not covalently bound to amino groups, still interacts with hydrophobic parts of the rod outer segment membrane through van der Waals forces. The question whether there is only one lysine residue providing its ε-amino group for the retinaldehyde binding or several amino groups one after another, will be discussed in the next chapter.

CHAPTER 4

TRANSIMINIZATION OF THE CHROMOPHORE

4.1 INTRODUCTION

Transiminization of retinylidene aldimines occurs relatively readily (Daemen, Jansen and Bonting, 1971). Such a transiminization could also occur somewhere during the photolytic sequence. The conversion from rhodopsin to metarhodopsin I is so rapid that such a transiminization seems unlikely. However, there is a definite possibility that it might occur during the conversion of metarhodopsin I to metarhodopsin II, since this reaction occurs in the order of a millisecond, requires water and has a high activation enthalpy and a high activation entropy change. If this would be the case, it could be of great importance for the mechanism of visual excitation. Even if transiminization would occur after metarhodopsin II formation, it may still have a functional role. The amino groups involved in this transiminization may constitute a pathway along which the retinaldehyde migrates from the chromophore binding site to other sites, e.g. the active center of retinoldehydrogenase or isomerase.

To determine whether transiminization occurs, we "fixed" the chromophoric group to its binding site by treatment with $NaBH_4$ during or after illumination and then "probed" the original chromophoric binding site by incubation with 11-cis retinaldehyde. If this yields a photopigment, it would mean that the original chromophoric site in the opsin molecule was available for the recombination reaction and was not occupied by a retinylgroup. In this case transiminization must have occurred

prior to reduction (fig.20). If however, no transiminization has occurred, the retinylgroup would still occupy the chromophoric site and no photopigment could be formed by incubation with 11-cis retinaldehyde.

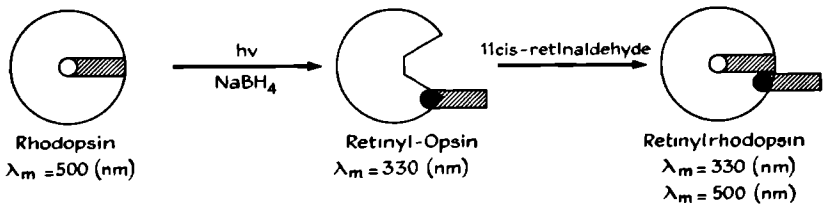

Fig 20. Formation of retinylrhodopsin upon incubation of illuminated and reduced rhodopsin with 11-cis retinaldehyde (O aldimine bond at original binding site, ● reduced aldimine bond). This reaction can only occur, if the chromophore migrates to a new binding site prior to reduction.

4.2 METHODS

4.2.1 Treatment with $NaBH_4$

The first photolytic product, which can be reduced by $NaBH_4$, is metarhodopsin II. In order to reduce photolyzed rhodopsin in the metarhodopsin II stage, 2 mg $NaBH_4$ was added to 1 ml of a 25 μ M rhodopsin suspension in 0.25 M phosphate buffer (pH 6.7) 5 seconds prior to illumination. Homogeneous distribution of the $NaBH_4$ was obtained by shaking the mixture vigorously before illumination. Reduction was observed qualitatively

(and visually) by a shift in the wavelength of maximal absorbance from 500 nm to 330 nm.

Quantitative determination of the degree of reduction was carried out by measuring the decrease in color formation with thiobarbituric acid. The retinyl group in retinylopsin gives no color with thiobarbituric acid, as was demonstrated with retinyllysine (prepared according to Daemen, Jansen and Bonting, 1971). Samples of 200 µl of the $NaBH_4$-treated and non-treated, illuminated rhodopsin suspension, were centrifuged at 18,100 x g for 5 min. After removal of 150 µl of the supernatants, 150 µl thiobarbituric acid reagent was added to the pellets plus remaining supernatant. The mixtures were homogenized and kept in the dark for 30 min. The resulting chromogen, which is relatively stable since the mixture contains no more than 10% water, was measured at 530 nm. The percentage reduction was calculated by dividing the retinaldehyde content (A_{530}) of photolyzed rhodopsin before and after $NaBH_4$-reduction. The concentration of retinylopsin was determined from the percentage reduction and the amount of rhodopsin present before reduction (calculated from ΔA_{500} and the molar absorbance).

4.2.2 Reaction with 11-cis retinaldehyde

Since the reaction between opsin and 11-cis retinaldehyde proceeds optimally at pH 6.3, the pellet of reduced photolyzed rhodopsin obtained after centrifugation was resuspended in a 0.067 M phosphate buffer pH 6.3. It was incubated for 3 hours after the addition of a 4-fold molar excess of 11-cis retinaldehyde, which represents optimal conditions for regeneration (section 3.2.5.). After incubation the excess retinaldehyde was removed

by hexane extraction and absorption spectra of the reaction mixture before and after illumination were determined. The amount of photopigment formed was determined from the decrease in absorbance at 500 nm upon illumination and is expressed in percent of the amount of rhodopsin originally present in the preparation.

4.3 RESULTS

4.3.1 Initial experiments

At pH 8.0, reduction of metarhodopsin II was fast and complete both in 0.25 M phosphate buffer and in the presence of 1% digitonin (fig.21). At pH below 8.0 the reduction proceeded less easily, especially in digitonin solution. Since however the brief exposure to pH 8.0 during reduction might have a denaturing effect on the retinylopsin, rendering it less capable to react with 11-cis retinaldehyde, we performed the reduction at pH 6.7. At this pH reduction in 1% digitonin solution was no longer possible, but fortunately photolyzed rhodopsin suspended in 0.25 M phosphate buffer could still be converted to retinylopsin for 80-90%. Analysis of the hexane extract of the reduction product by thin-layer chromatography showed only trace amounts of retinol and no retinylphospholipids. Only the residue, remaining after extraction, displayed fluorescence, indicating that the retinylgroup must be bound to the protein part of the opsin molecule. A spectrophotometric retinol determination in the extract with Carr-Price reagent showed that less than 4% of the chromophoric retinaldehyde was released and reduced to retinol.

In the early experiments we used an aged preparation of $NaBH_4$, which later was found to be partly hydrolyzed.

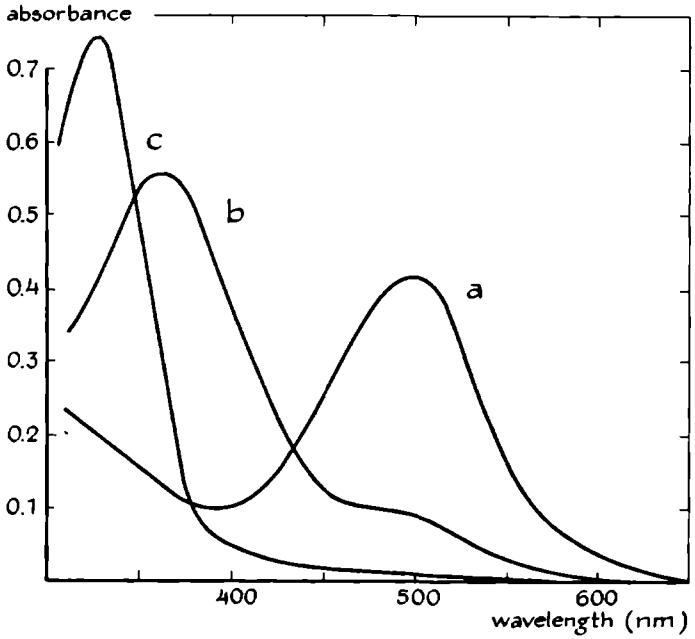

Fig 21. Spectra of rhodopsin (a), illuminated rhodopsin (b) and rhodopsin illuminated in the presence of $NaBH_4$ (c). Reduction was in phosphate buffer (pH 8.0). Spectra were measured after solubilization in Triton-X-100.

When this $NaBH_4$ was added to the rhodopsin suspension a few seconds before illumination, the reduction of photolyzed rhodopsin was rather slow, taking about 1 min. Apparently, the non-reducing outer shell of these $NaBH_4$ grains had to dissolve first. Incubation of reduced photolyzed rhodopsin with 11-cis retinaldehyde clearly led to the formation of a substantial amount of photopigment. The

spectrum of the regenerated pigment was measured after removal of the excess retinaldehyde by repeated hexane extraction. The absorption spectrum displayed maxima at 330 and 500 nm. The peak at 500 nm can be ascribed to 11-cis retinaldehyde bound by means of an aldimine link to the chromophoric site, while the 330 nm peak must be due to the retinyl group resulting from reduction of the migrated original chromophoric group, now bound by a secundary amine link to another amino group. Illumination caused a decrease in absorbance at 500 nm and an increase at 360 nm (fig 22), much as is the case for rhodopsin.

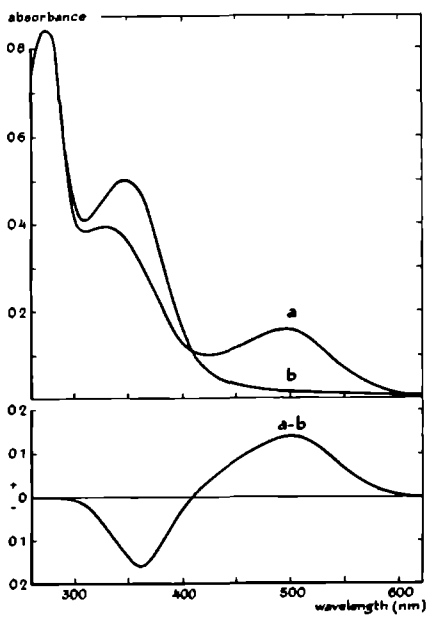

Fig 22. Photolysis of retinylrhodopsin, prepared from $NaBH_4$-reduced photolyzed rhodopsin and 11-cis retinaldehyde. The absorption spectra were measured after solubilization in 1% Emulphogene before (a) and after (b) illumination. The difference spectrum is depicted below by a-b.

To calculate the amount of photopigment formed, we first determined whether the presence of a retinyl group had changed the molar absorbance at 500 nm. The molar absorbance of the photopigment formed was calculated from the retinaldehyde concentration (determined by thiobarbituric acid) and the absorbance at 500 nm. The photopigment had nearly the same molar absorbance (39000) as untreated rhodopsin (40300 $\pm$ 500), which means that the presence of the retinyl group has no effect on the molar absorbance at 500 nm. Thus, we could calculate the total pigment concentration as fraction of the rhodopsin originally present (table II, column 2) by means of ΔA_{500}. Since the recombination reaction need not be complete, we always incubated in parallel a control sample of photolyzed rhodopsin, which had not been treated with $NaBH_4$ (table II, column 3). Taking as an example the first experiment (79% reduction, 37% regeneration in the reduced sample and 51% regeneration in the non-reduced control sample), 21% of the originally present rhodopsin was not reduced, which should give 0.51 x 21 = 11% rhodopsin (table II, column 4), assuming an equal percentage regeneration in the $NaBH_4$-treated sample and the control sample. Subtracting this percentage from the total percentage regeneration (37%), we conclude that 26% (table II, column 5) of the original amount of rhodopsin must have been converted to retinylrhodopsin. Relating this percentage to the percentage reduction (79%), i.e. the percentage retinylopsin formed during reduction, we conclude that 26/0.79 = 33% (table II, column 6) of the retinylopsin has thus been converted, in other words has bound 11-cis retinaldehyde to its chromophoric site.

The results of table II indicate that a large amount of a new photopigment, which we have called retinyl-

Table II. Formation of retinylrhodopsin after delayed reduction with $NaBH_4$. All experiments were performed in phosphate buffer; reduction took place within 1.2 minutes after photolysis.

1	2	3	4	5	6
0.79	0.37	0.51	0.11	0.26	0.33
0.49*	0.90	1.00	0.51	0.39	0.80
0.80	0.71	0.95	0.19	0.52	0.65
0.92	0.72	0.65	0.05	0.67	0.73

*Low reduction percentage due to shortage of $NaBH_4$.

Meaning of columns:

1. fraction of rhodopsin, which is reduced after photolysis.
2. fraction of rhodopsin, which after photolysis and reduction forms with 11-cis retinaldehyde photopigment.
3. fraction of rhodopsin in control sample (no $NaBH_4$), which after photolysis forms with 11-cis retinaldehyde rhodopsin.
4. calculated fraction of rhodopsin, which is not reduced and has formed with 11-cis retinaldehyde rhodopsin.
5. calculated fraction of rhodopsin, which is reduced and has formed with 11-cis retinaldehyde retinylrhodopsin.
6. calculated fraction of retinylopsin, which forms with 11-cis retinaldehyde retinylrhodopsin.

rhodopsin, must have been formed by the reaction of retinylopsin with 11-cis retinaldehyde. This proves that in these experiments a large part of the isomerized chromophore has left the chromophoric site within 1-2 minutes after illumination, but is still bound to other amino groups in the rod outer segment preparation where its link is reduced and stabilized by $NaBH_4$.

4.3.2 Immediate reduction

The experiments reported in the pervious section do not give a decisive answer to the question, whether transiminization has occurred during or immediately after metarhodopsin II formation, since reduction was complete only 1-2 minutes after formation of this intermediate. The time needed for complete reduction could be substantially shortened by using $NaBH_4$ of high purity (Fluka, Switzerland). No accumulation of the yellow metarhodopsin II was observed when rhodopsin was illuminated in the presence of this $NaBH_4$-preparation. Complete decoloration was achieved already after a few seconds; reduction was complete for 90-95% within 5 sec. After incubating the reduced metarhodopsin II for 3 hrs with a 4-fold excess of 11-cis retinaldehyde, no photopigment was formed since illumination caused no significant change in the absorbance at 500 nm. This indicates that no significant amount of retinylrhodopsin was formed, when metarhodopsin was reduced within 5 sec of its formation. Under the same conditions non-reduced photolyzed rhodopsin did react with 11-cis retinaldehyde. In order to exclude the possibility that the loss of regeneration capacity of reduced metarhodopsin II could be due to changes in the protein structure caused by the action of $NaBH_4$, we treated non-photolyzed rhodopsin with a large excess

$NaBH_4$ in darkness. After an incubation period of 10 minutes, the excess $NaBH_4$, was washed away, the preparation was photolyzed and was incubated with 11-cis retinaldehyde. Exactly the same regeneration percentages were obtained with rhodopsin treated with $NaBH_4$ in darkness and with rhodopsin not treated with $NaBH_4$. The same results were obtained with retinaldehyde-free opsin. Thus, any change in protein structure which might arise from the action of $NaBH_4$, does not influence the reaction with 11-cis retinaldehyde.

The inability of retinylopsin to react with 11-cis retinaldehyde must be caused by the reduction of a particular aldimine bond. This is indicated by the following experiment. A rhodopsin preparation was incubated with 5 moles added all-trans retinaldehyde per mole of non-photolyzed rhodopsin in order to form some random aldimine bonds. After treatment with $NaBH_4$, the regeneration capacity of the preparation had not changed.

4.3.3 Delayed reduction

In subsequent experiments we systematically enlarged the time span between photolysis and reduction in order to investigate whether the formation of retinylrhodopsin would then increase and how this would relate to the known release of retinaldehyde. These experiments showed a rise in regeneration capacity from 0 to 90% when the time interval between photolysis and reduction increased from 0 to 60 min (fig. 23). Since the release of retinaldehyde was much less at all intervals, this proves that transiminization of the chromophore must take place after illumination. The course of the increase of the regeneration capacity with time varies considerable with the preparation used.

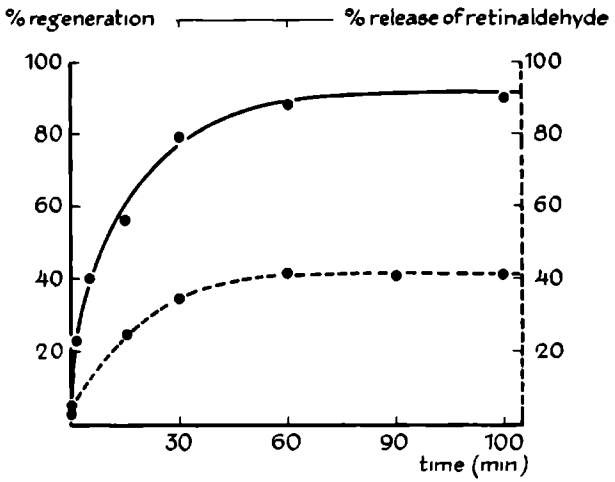

Fig 23. Regeneration capacity of reduced photolyzed rhodopsin as a function of the time interval between illumination and reduction. The regeneration capacity of the non-reduced sample was 100%. The dashed curve represents the hydrolysis of retinylidene-opsin. (see fig 19). Both experiments were carried out at 25°C.

These differences in the rise of regeneration capacity are very likely due to a different rate of transiminization in different preparations. Some preparations seem to loose the structural conditions necessary for transiminization more rapidly than other preparations. It appears that there are quite distinct steps in rhodopsin denaturation, leading to a loss of transiminization capacity first, than to a loss of regeneration capacity and finally to

a loss of the 500 nm absorbance.

The regeneration capacity was never completely regained. This cannot be due to a lower stability of retinylopsin as compared to that of opsin, since the regeneration percentages for retinylrhodopsin and resynthesized rhodopsin are equal (88% on the average). Much more likely is that the chromophoric amino group remains partially blocked after migration of the chromophoric retinaldehyde.

4.4 DISCUSSION

A water-soluble enzyme is independent from its substrate until a collision leads to the formation of an enzyme-substrate complex. In the case of rhodopsin, however, the chance of enzymatic reduction (retinoldehydrogenase) or isomerization (isomerase) of all-trans retinaldehyde, and of recombination of opsin and 11-cis retinaldehyde, might be rather low in the closely packed, well organized membranes of the rod outer segment, when no specific "routes" for these retinaldehyde isomers would be present. Moreover, the lipophilic character of retinaldehyde makes it rather difficult to assume that this substrate would go into "solution" and would only be reduced or isomerized after a chance collision with the active site of retinoldehydrogenase or isomerase molecules.

A migration of retinaldehyde by consecutive transiminization reactions is very well possible, since the rod outer segment membranes contain some 16 lysine residues and some 36 amino group containing phospholipids per rhodopsin molecule. To determine whether this transiminization occurs during metarhodopsin II formation, we incubated reduced metarhodopsin II with 11-cis retinaldehyde in order to "probe" the chromophoric site. Since metarhodopsin II decays rather rapidly, reduction had to be carried out as fast as possible in order to determine whether migration

takes place during its formation. When in our initial experiments $NaBH_4$ of rather poor quality was used, complete reduction took about 1 min and a considerable amount of photopigment was formed upon subsequent incubation with 11-cis retinaldehyde. When, however, high quality $NaBH_4$ was used, reduction was complete within 5 seconds and no photopigment was obtained after incubation with 11-cis retinaldehyde. This clearly indicates that no chromophore migration takes place during the formation of metarhodopsin II. We could show that the absence of photopigment formation was not due to a loss of regeneration capacity upon treatment with $NaBH_4$, since the regeneration capacity of native rhodopsin and retinaldehyde-free opsin was not affected by $NaBH_4$-treatment. Even reductive fastening of a three-fold molar amount of added all-trans retinaldehyde did not change the regeneration capacity. These experiments show that the $NaBH_4$-treatment in itself or the reductive fastening of chromophore elsewhere on the opsin molecule cannot explain the absence of regeneration capacity after reduction within 5 seconds. Rather the reduction of a particular retinaldehyde-opsin linkage namely the original linkage, must be responsible.

This particular bond must, therefore, be the original bond between retinaldehyde and the "chromophoric" lysine residue. Thus, transiminization does not seem to occur during the conversion from metarhodopsin I to metarhodopsin II, which would mean that the chromophore in metarhodopsin II is bound to the same lysine residue as in rhodopsin. This conclusion is supported by the induced circular dichroism studies of Waggoner and Stryer (1971). These authors found a great similarity of induced circular dichroism of rhodopsin, metarhodopsin I and metarhodopsin II, which suggests that the dissymmetric interactions at the chromophore binding site are nearly the same in these three species. Thus, a

significant portion of the local environment of the retinaldehyde is conserved in the transition from rhodopsin to metarhodopsin I to metarhodopsin II. In all fairness, it must be admitted that our experiments cannot exclude transiminization to a site so near that this would through steric hindrance shield the original binding site from the added 11-cis retinaldehyde.

Upon enlargement of the time span between photolysis and reduction beyond 1 minute, formation of retinylrhodopsin took place in increasing amount. This implies that the original chromophoric site was becoming available to 11-cis retinaldehyde. There are no other sites in the rod outer segment preparation which yield a rhodopsin-like pigment after incubation with 11-cis retinaldehyde, since the ΔA_{500} of native rhodopsin cannot be increased by incubation with 11-cis retinaldehyde (de Grip, Daemen and Bonting, 1972).

The observed increase in availability of the chromophoric site is faster and more extensive than the release of retinaldehyde. Therefore, transiminization after metarhodopsin II formation must have been occured. In chapter 5 experiments will be described which show that the active site of the enzyme retinoldehydrogenase is the "receiving site" for the all-trans retinaldehyde.

CHAPTER 5

RETINOLDEHYDROGENASE ACTIVITY IN ROD OUTER SEGMENT MEMBRANES

5.1 INTRODUCTION

The final product of bleaching in vitro is a mixture of retinaldehyde and opsin (Wald, 1938; Bridges, 1962[a]), for the most part still loosely coupled together (Wald, 1949) or even covalently bound (see chapter 3). In vivo, the major part of the retinaldehyde is converted to retinol (Wald, 1935[b]) suggesting that the rod outer segment membranes contain retinoldehydrogenase activity. In a preparation of fresh isolated rods, retinol is the end product of photolysis, but in aged or washed preparation, no reduction of retinaldehyde occurs unless NADH is added (Bridges, 1962[b]). Futterman (1963) discovered that NADPH was a better coenzyme than NADH for this reaction, which has been confirmed by de Pont, Daemen and Bonting (1970[a]).

The enzymatic reduction of free retinaldehyde is catalyzed by dehydrogenases present in the retina (Wald, 1949 and 1950; Wald and Hubbard, 1949; Futterman and Saslaw, 1961; Futterman, 1963 and 1965; Koen and Shaw, 1966; Reading and Sorsby, 1966; de Pont, Daemen and Bonting, 1970). The reduction is also catalyzed by horse liver alcoholdehydrogenase (Bliss, 1948, 1949 and 1951[a]; Zachman and Olsen, 1961; Futterman and Saslaw, 1961; Futterman, 1963 and 1965, Koen and Shaw, 1966; de Pont, Daemen and Bonting, 1970).

Koen and Shaw (1966) reported the presence of two retinal isoenzymes of retinoldehydrogenase and one retinal alcoholdehydrogenase, the latter being electroforetically distinct from hepatic alcoholdehydrogenase. The fundamental difference between the liver alcoholdehydrogenase and the retinol-

dehydrogenase of the retinal rods is further supported by de Pont, Daemen and Bonting (1970[a]), who showed that the retinal retinoldehydrogenase is capable of reducing retinylidene-amines to free retinol and amine, whereas the hepatic enzyme is not. The hepatic and retinal alcoholdehydrogenases are chemically different, since they had different substrate K_M-values (Watkins and Tephly, 1971). Furthermore, the retinal alcoholdehydrogenase was not inhibited by 1,10-phenanthroline and was not able to oxidize retinol. The metabolic role of the retinal alcoholdehydrogenase is unknown.

The studies of Koen and Shaw (1966) have involved rather crude preparations of whole retina, which certainly contained both soluble and particulate dehydrogenases. The occurrence of two retinal retinoldehydrogenases cannot be stablished with certainty in these crude preparations. De Pont, Daemen and Bonting (1970[a]) showed that after ultracentrifugation of a rod outer segment preparation all retinoldehydrogenase activity is located in the pellet, which suggests that in the rod sac membrane a retinoldehydrogenase is present. The retinoldehydrogenase preparations used in our study were washed several times before use in order to remove any soluble dehydrogenases, and thus represent the same enzyme as that in the study of the Pont et al.

So far most studies on retinoldehydrogenases have been performed in detergent solution. The action of detergents on the retinoldehydrogenase enzyme should be known before one can consider possible methods of purification. It is rather likely that the action of these detergents is not confined to solubilization, but involves also a certain degree of denaturation. We have therefore,investigated whether retinoldehydrogenase preparations solubilized in detergent solutions behave differently from preparations suspended in phosphate buffer.

Moreover, most previous studies on retinoldehydrogenase were performed with a large excess of exogenous retinaldehyde. In these experiments the enzyme was saturated with substrate, so that steady state kinetics could be applied. In physiological circumstances, however, there will not be such an excess of substrate. Therefore, we decided to investigate the enzymatic reduction of endogenous retinaldehyde, because the properties of the enzyme may depend on the substrate concentration. In these experiments, an illuminated rod outer segment preparation serves both as enzyme and as substrate. The enzymatic conversion starts after the addition of NADPH, which acts as coenzyme.

The use of suspensions of rod outer segments enabled us to determine the nature of the intermediary product of enzymatic reduction and the relationship between the retinoldehydrogenase system and the chromophore binding site.

5.2 MATERIALS AND METHODS

5.2.1 Enzyme preparation

Rod outer segment preparations were obtained as described in 2.2.1. The material was prepared in dim red light. After lyophilization, the preparation was stored at -70^{o}C in the dark until further use. About 10 mg dry rod outer segment preparation was suspended in 0.5 ml 0.067 M phosphate buffer (pH 6.5) by homogenization in a Potter-Elvehjem tube. In certain experiments a detergent was added to the phosphate buffer to a concentration of 1% (w/v). In some cases this suspension, with or without detergent, was used as the enzyme preparation, but usually the preparation was illuminated first. Retinaldehyde-free opsin, which was also used as enzyme preparation, was

prepared as described in 3.2.1.

5.2.2 Reduction by $NaBH_4$

To 1 ml of a rod outer segment suspension (25 nmol rhodopsin)in 0.25 M phosphate buffer (pH 7.0), 2 mg $NaBH_4$ (Fluka, Switzerland) was added. The suspension was vigorously mixed for a few seconds and kept in the dark for 15 min. A second sample was illuminated by three 300 W tungsten lamps for 5 min at 15 cm distance without filters immediately after the addition of the $NaBH_4$. This high intensity of illumination was necessary to secure rapid and complete bleaching, which was necessary to retain full enzyme activity after immediate $NaBH_4$ treatment (see section 5.3.4). Other samples were treated with $NaBH_4$ various times after illumination. After illumination and reduction, the samples were kept in the dark for 15 min. The insoluble reduced rod outer segment material was separated from the excess $NaBH_4$ by centrifugation (35,000 x g, 15 min). The pellet was resuspended in 0.25 M phosphate buffer (pH 6.0) and centrifuged again (35,000 x g, 15 min). The resulting pellet was taken up in 200 µl 0.067 M phosphate buffer (pH 6.0). The percentage reduction was calculated from thiobarbituric acid determinations of retinaldehyde content in samples of illuminated rhodopsin before and after reduction. The reduction was always between 95 and 100% complete.

5.2.3 Retinoldehydrogenase assay

Various media were used (CTAB, Triton, Emulphogene, phosphate buffer), but the amount of rod outer segment material in 200 µl medium always contained about 25 nmol rhodopsin, photolyzed rhodopsin, retinylopsin or opsin.

A suspension of illuminated rod outer segments acts both as enzyme and as substrate. When a non-illuminated rod outer segment suspension, a $NaBH_4$-reduced illuminated rod outer segment suspension or a retinaldehyde-free opsin preparation was used, a two-fold molar amount of exogenous all-trans retinaldehyde (50 nmol), dissolved in 10 µl acetone, was added. When the enzyme preparation consisted of rhodopsin, retinylopsin or opsin, parallel incubations were performed with photolyzed rhodopsin. The enzymatic reduction was started by the addition of a five-fold molar amount of NADPH (125 or 250 nmol). All incubations were conducted at 37°C. In some experiments the all-trans retinaldehyde was replaced by the 11-cis isomer (see 2.2.2).

The amount of substrate reduced was determined by withdrawing 25 µl samples prior to the addition of all-trans retinaldehyde, prior to the addition of NADPH and at various times after the addition of NADPH. The samples were added to 300 µl thiobarbituric acid reagent. After 30 min in the dark the samples were centrifuged (30,000 x g, 10 min) and the absorbance at 530 nm of the supernatant was measured. After applying corrections for changes in volume due to sample taking prior to NADPH addition, the initial rate of enzymatic reduction was calculated from the decrease in the retinaldehyde concentration. Concentrations of acetone, which are used in the solubilization of retinaldehyde, did not inhibit the enzyme reaction.

5.2.4 Enzymatic oxidation of retinol

The all-trans retinol, formed upon incubation of photolyzed rhodopsin with a five-fold molar amount of NADPH, was used as the substrate for the reserve reaction. In some experiments an additional equivalent of exogenous all-trans retinol was added. All incubations were carried

out at 37° under nitrogen. After addition of a 20-fold molar amount of $NADP^+$, the increase in retinaldehyde concentration was measured by means of the thiobarbituric acid method. The 11-cis retinol was prepared by $NaBH_4$-reduction of 11-cis retinaldehyde and its purity was checked by thin-layer chromatography (ether-hexane; 1:1 v/v).

5.3 RESULTS

5.3.1 Retinoldehydrogenase activity in various media

The action of retinoldehydrogenase on photolyzed rhodopsin results in a conversion of retinaldehyde to retinol, while the coenzyme NADPH is simultaneously oxidized to $NADP^+$. Control experiments showed that in the absence of NADPH no decrease in retinaldehyde concentration occurs (fig 24). When the illuminated rod outer segment preparation is replaced by an equivalent amount of free all-trans retinaldehyde in the absence of NADPH, a significant decrease in concentration was observed. This suggests that the retinaldehyde is protected from oxygen by lipophilic interactions within the rod outer segment preparation. In principle, the conversion rate can be calculated from determinations of each of the four components participating in the reaction, in practice determination of the decrease in retinaldehyde concentration by means of thiobarbituric acid determinations gives the most accurate results.

We investigated the enzymatic reduction in phosphate buffer, in the non-ionic detergents digitonin, Triton-X-100 and Emulphogene and in the cationic detergent cetyltrimethylammoniumbromide (CTAB). The initial reaction rate was always highest in phosphate buffer. No decrease in activity was observed after storage of a non-illuminated

rod outer segment suspension for 24 hours at 20°C.

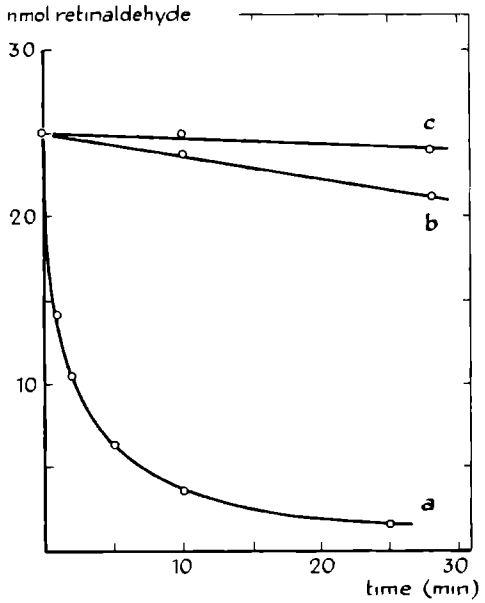

Fig 24. Enzymatic reduction of retinaldehyde. Illuminated rod outer segments, containing 25 nmol endogenous retinaldehyde, are suspended in 200 µl 0.067 M phosphate buffer (pH 6.0). After 1 min preincubation at 37°, 125 nmol NADPH is added. The decrease in retinaldehyde content is measured by the thiobarbituric acid method (a). When NADPH is absent, nearly no decrease is observed (c). Free all-trans retinaldehyde is slowly degradated by oxygen (b).

In digitonin solution the activity dropped to 80% after 6 hours and to 60% after 24 hours at 20°C. The other detergents caused more rapid denaturation of the enzyme. In Emulphogene and Triton-X-100 the activity fell to

70-80% in 10 min and to 15-25% after 6 hours. In CTAB the activity was nearly completely lost within 10 min. The decrease in activity in detergent solutions cannot be prevented by addition of NADPH or all-trans retinaldehyde. These results are summarized in table III.

Table III. Stability of retinoldehydrogenase (at 20°C) in various media. The initial rate in suspension, set at 100%, does not change during the 6 hour period.

time in detergent / detergent	10 min	30 min	6 hours
None	100%	100%	100%
Digitonin	100%	100%	80%
Triton-X-100	70%	45%	25%
Emulphogene	80%	45%	15%
CTAB	5%	0%	0%

5.3.2 Enzymatic reduction of retinaldehyde

In further experiments a suspension of rod outer segments in 0.067 M phosphate buffer was used as retinoldehydrogenase preparation.

When equal amounts of NADPH and photolyzed rhodopsin were incubated, an equilibrium was reached in which about 40% of the retinaldehyde was reduced. The equilibrium was

only slightly dependent on the pH between pH 5 and 8. In vivo the ratio between free retinol and retinaldehyde in a light-adapted retina is about 3.4 (Futterman, 1965).

When a five-fold molar amount of NADPH was used, a nearly complete reduction of all-trans retinaldehyde occurred. An increase in the NADPH/retinaldehyde ratio beyond 5:1 did not further alter the reaction rate. In these experiments photolyzed rhodopsin was used as substrate, so the substrate is present in a physiological concentration. This may mean that the enzyme is far from being saturated with substrate. We merely compared the initial reaction rates and the overall course of the enzymatic reduction.

Between pH 5.0 and 6.5, the reaction course is about the same. Beyond this pH range the reaction slows down and the reduction is less complete (fig 25). Incubation with NADH instead of NADPH causes a decrease in reaction rate. This result confirms the studies of Futterman (1963) and de Pont, Daemen and Bonting (1970) on the coenzyme dependence of the retinoldehydrogenase system. At pH 8, the activity decreases by 80%, while at pH 5 only a 10% decrease was observed, when NADPH is replaced by NADH.

When the rod outer segment suspension is not illuminated, the endogenous retinaldehyde is tightly bound to the chromophoric site and cannot be reduced enzymatically. In this case exogenous retinaldehyde can serve as substrate. All-trans retinaldehyde, incubated with a dark-adapted rod outer segment preparation and a five-fold molar amount of NADPH, is nearly completely converted to retinol. The initial reaction rate obtained after incubation of an illuminated rod outer segment preparation with its endogenous retinaldehyde, is equal to the rate obtained after incubation of the same preparation in the dark with an equivalent amount of exogenous all-trans retinaldehyde. According to fig 24 this initial rate is 25 nmol

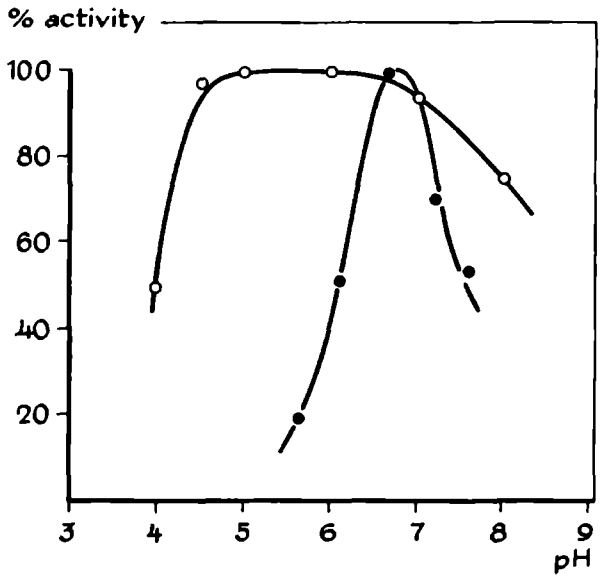

Fig 25. Dependence on pH of the retinoldehydrogenase activity in suspension (o-o) and in 1% Triton-X-100 (x-x). The latter experiment is from the Pont, Daemen and Bonting (1970[b]). Maximal activity is set at 100% in both experiments.

retinaldehyde per 2 min per 25 nmol rhodopsin or 0.25 mole per kg dry weight per hour. When an illuminated rod outer segment preparation is incubated with an equimolar amount of all-trans retinaldehyde, the initial rate is almost exactly twice as high as without the addition of the extra amount of

substrate. So, in this concentration range, the initial rate is proportional to the concentration of retinaldehyde, regardless whether endogenous or exogenous retinaldehyde is used. The maximal activity was estimated from a Lineweaver-Burk plot to be about 1.1 mole per kg dry weight per hour. When NADH is used instead of NADPH, exogenous retinaldehyde is converted somewhat slower than endogenous retinaldehyde.

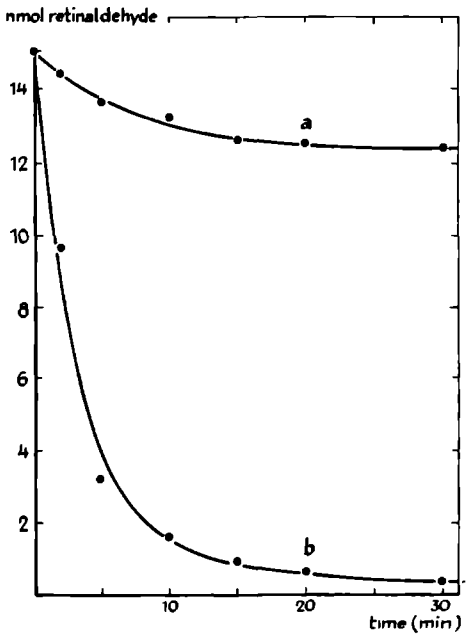

Fig 26. Stereospecificity of enzymatic reduction of retinaldehyde. A suspension of dark adapted rod outer segments in 1 ml 0.067 M phosphate buffer (pH 6.0), containing 7 nmol rhodopsin, was incubated in the dark at 25°, with 15 nmol 11-cis retinaldehyde (a) or with 15 nmol all-trans retinaldehyde (b).

All-trans retinaldehyde is the best substrate for the retinoldehydrogenase enzyme. The 11-cis isomer is reduced only very slowly (fig 26). The slow conversion could very well be caused by a slow isomerization to the all-trans isomer (see chapter 3), which is then reduced. It might, therefore, be very well possible that the 11-cis isomer is not reduced at all. This implies a high degree of stereo-specificity of the enzyme.

5.3.3 Enzymatic oxidation of retinol

The oxidation of retinol to retinaldehyde, which occurs in the intact eye and can also be demonstrated in vitro (Wald and Hubbard, 1950 ; Muirhead, 1967; Watkins and Tephly, 1971), proceeded also in our purified rod outer segment preparations. Under optimal conditions (0.1 M tris-HCl buffer pH 8.0; $NADP^+$ as coenzyme), enzymatic oxidation of endogenous retinol is only about 40% complete (fig 27). The low yield of the enzymatic oxidation is due to a partial loss of substrate through non-specific oxidation. After increasing the substrate concentration by addition of an extra equivalent all-trans retinol, the amount of retinaldehyde produced is more than doubled. The non-specific destructive oxidation of retinol is known to occur more rapidly in colloidal dispersion than in true solution (Lucy, 1966). At the very low retinol concentrations used, it is very difficult to avoid the presence of molar excess of oxygen even though we used a nitrogen atmosphere.

The all-trans isomer of retinol seems to be the best substrate for enzymatic oxidation, although oxidation of 11-cis retinol does occur. We have not investigated whether this oxidation is made possible by non-specific isomerization to all-trans retinol. This seems rather likely in view of the flatter curve for 11-cis retinol.

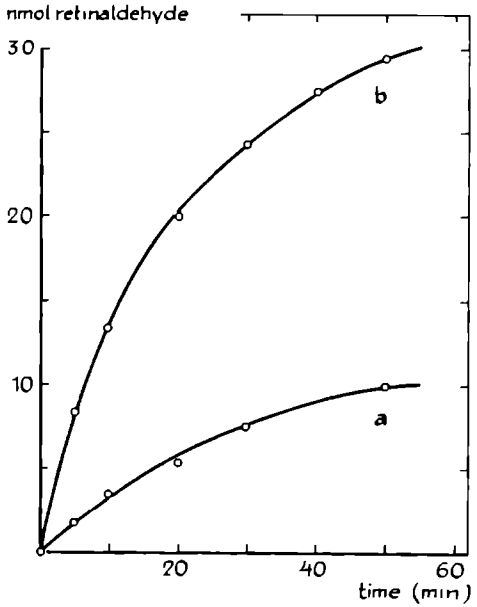

Fig 27. Enzymatic oxidation of retinol. Retinol formed upon enzymatic reduction of 25 nmol retinaldehyde (125 nmol NADPH as coenzyme) is used as substrate. Immediately after complete conversion of the retinaldehyde (taking about 30 min), the preparation is transferred to 200 µl 0.067 M phosphate buffer (pH 8.0) and 500 nmol $NADP^+$ is added. The increase in retinaldehyde content is measured by the thiobarbituric acid method (a). In (b) 25 nmol all-trans retinol is added to the incubation mixture.

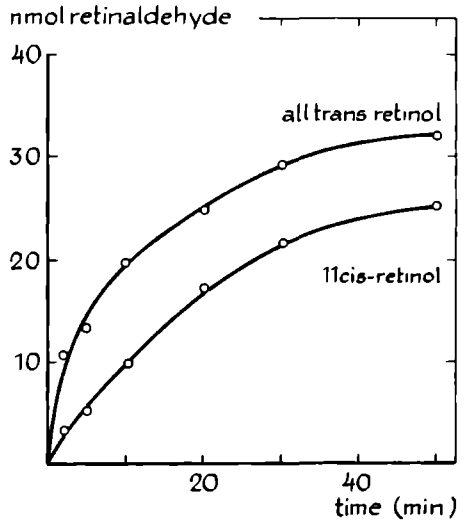

Fig 28. Stereospecificity of enzymatic oxidation of retinol. After 1 min preincubation of a suspension of dark-adapted rod outer segments (containing 25 nmol rhodopsin) in 0.067 M phosphate buffer (pH 8.0), 50 nmol all-trans retinol or 50 nmol 11-cis retinol are added. After addition of 250 nmol $NADP^+$, the increase in retinaldehyde content is measured.

5.3.4 $NaBH_4$-reduced photolyzed rod outer segments

The localization of the retinoldehydrogenase site and its relation to the chromophoric site are still completely unknown. Connected with this is the question how many retinoldehydrogenase sites are present per

chromophoric site. Unless the retinoldehydrogenase site would be part of the opsine molecule, it seems rather unlikely that the retinoldehydrogenase sites are present in an equivalent number as the chromophoric sites, since in that case a very large overcapacity of enzyme would be present. In order to obtain a closer insight into the relation between these two sites, both sites were chemically modified by reductive fastening of the endogenous retinaldehyde to amino groups in the rod outer segment preparation. The retinaldehyde in metarhodopsin II or in later photolytic products can be fastened to the opsin molecule by $NaBH_4$, which reduces the labile aldimine bond to a stabile secondary amine link (retinyl-opsin).

When the retinaldehyde is reduced while it is bound to the chromophore-carrying amino group, no regeneration of photopigment can occur upon addition of 11-cis retinaldehyde (see chapter 4). Similarly, it is also obvious that retinaldehyde linked by means of a secondary amine bond to opsin is not available as a substrate to the retinoldehydrogenase. The physiological substrate can however be replaced by exogenous retinaldehyde, but this will only be reduced when the active site of retinoldehydrogenase remains intact. Since an amino group in the active site might serve to bind the aldehyde substrate, $NaBH_4$ treatment might very well cause a reductive fixation of endogenous retinaldehyde on the active site. It would covalently attach retinaldehyde to this specific amino group and this would certainly abolish the enzymatic activity. Hence, a possible binding of retinaldehyde to an amino group of the retinoldehydrogenase site should be detected by measurement of the retinoldehydrogenase activity after reduction with $NaBH_4$.

$NaBH_4$-reduction of the retinaldehyde-opsin linkage in native rhodopsin is not possible, presumably due to

inaccessibility of this linkage to the reductant. Reduction immediately after photolysis leads to fixation on the chromophore-carrying site, since the resulting retinylopsin does not form a photopigment upon incubation with 11-cis retinaldehyde (chapter 4.3.2). The retinol-dehydrogenase activity after photolysis in the presence of $NaBH_4$ is not reduced. With increasing time intervals between photolysis and reduction, the retinoldehydrogenase activity decreases, until a minimum of about 20% is obtained at a time interval of 5-15 min between illumination and reduction. When this time-interval is increased to 30-60 min, a partial and variable restoration (30 to 80%) of the retinoldehydrogenase activity occurs. The variability in time needed for optimal occupation of the retinoldehydrogenase site and for the maximal restoration of activity, is due to differences in the rod outer segment preparation rather than to inaccuracies in the determination, since it does not occur in duplicate experiments performed with the same preparation.
A typical experiment is shown in fig 29. Treatment of native rhodopsin with $NaBH_4$ causes a decrease in retinoldehydrogenase activity. All these results are summarized in table IV.

Table IV. Effect of $NaBH_4$ treatment on the retinoldehydrogenase activity.

Native rhodopsin	100%
Illuminated rhodopsin	100%
Rhodopsin, reduced by $NaBH_4$ immediately after illumination	100%
Rhodopsin, reduced by $NaBH_4$ 5-15 min after illumination	20%
Rhodopsin, reduced by $NaBH_4$ 30-60 min after illumination	30-80%
Rhodopsin, treated in the dark with $NaBH_4$	55-75%

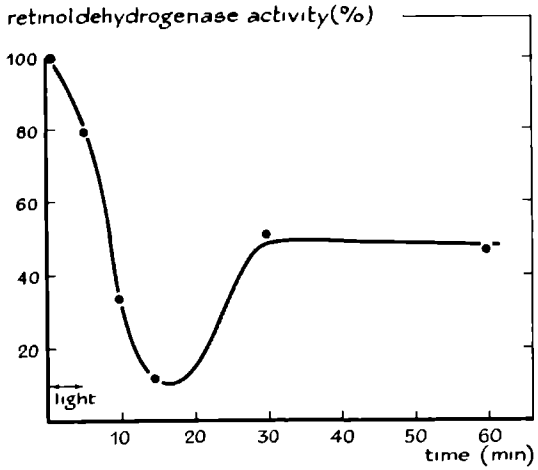

Fig 29. Retinoldehydrogenase activity as a function of the time span between illumination and reduction by $NaBH_4$. For experimental details, see text.

5.4 DISCUSSION

Destruction of the rod outer segment structure by osmotic shock or by homogenization causes no change in retinoldehydrogenase activity, and the enzyme remains insoluble. It must, therefore, be a part of the rod outer segment membrane.

The decrease in enzyme ectivity upon solubilization in detergents could either be due to denaturation of the enzyme molecule, or to loss of integrety of the molecular structure of the rod outer segment membrane. For the more drastic detergents Triton-X-100, Emulphogene and CTAB denaturation seems likely, since they also abolish the regeneration capacity of the rhodopsin. For digitonin, which does not affect pigment regeneration, the gradual

loss of retinoldehydrogenase activity may well be due to a loss of membrane integrety. The instability of the retinoldehydrogenase in detergents greatly hampers isolation of the enzyme from the rod outer segment membrane and its furhter purification. Up till now, most experiments on retinoldehydrogenase have been done in digitonin or Triton solutions. In view of the relative instability of the enzyme in these detergents, we decided to use mainly suspensions of rod outer segment membranes in our studies. Under these circumstances the enzyme is imbedded in the rod outer segment membrane, which should stabilize the enzyme and keep its properties close to those in vivo. The simulation of the in vivo situation was made even closer by using as substrate endogenous all-trans retinaldehyde instead of excess exogenous retinaldehyde.

When a suspension of rod outer segments is used as enzyme preparation, the enzymatic reduction is much less dependent on the pH than in detergent solution (fig 25). The hydrogen ion concentration around the active site apparently does not reflect the medium pH very well. A somewhat similar conclusion is reached by a consideration of the activity relative to the retinaldehyde concentration. We find an activity of 0.25 mole/kg dry weight/h, nearly the same as the activity of 0.30 mole/kg dry weight/h determined by de Pont, Daemen and Bonting (1970[a]) in Triton-X-100 with a retinaldehyde concentration of 0.83 mM, which activity was estimated to be about half the maximal rate. In our experiments in suspension, however, only 25 nmol retinaldehyde was available in an incubation volume of 200 µl representing an apparent substrate concentration of only 0.125 mM. This indicates that the actual substrate concentration around the active site must have been several times higher, presumably due to preferential localization of the retinaldehyde in

the lipophilic membrane structure. In detergent solution the activity would have been much lower at such apparent substrate concentration according to the results of de Pont, Daemen and Bonting (1970[a]). Both the pH effect and the substrate concentration indicate that in suspensions of rod membrane preparations the configuration around the active centre remains more intact than after solubilization in detergents.

The equality of the reaction rates for endogenous and exogenous retinaldehyde points to a rather easy accessibility of the active centre, at least at these low substrate concentrations. The active centre itself shows strong preference for the straight chain of the all-trans isomer. The enzymatic oxidation of all-trans retinol to retinaldehyde also proceeds faster than the oxidation of the 11-cis isomer.

The most suitable coenzymes for oxidation and reduction are $NADP^+$ and NADPH, respectively. Especially at high pH, the reaction rates are higher with $NADP^+$ and NADPH than with the non-phosphorylated coenzymes (NAD^+ and NADH). Under optimal conditions for each reaction the rate of reduction of all-trans retinaldehyde was about 5 times larger than the rate of enzymatic oxidation of all-trans retinol.

Retinoldehydrogenase activity is the same in native and photolyzed rhodopsin, indicating that occupation of the chromophoric site by 11-cis retinaldehyde has no influence on the enzymatic conversion of retinaldehyde. Therefore, this enzymatic reduction must occur at a site distinct from the chromophoric site.

$NaBH_4$-treatment of native rhodopsin causes partial inactivation of the enzyme activity, while it does not affect the regeneration capacity (chapter 4). Thus, in native rhodopsin the chromophoric site and the retinol-

dehydrogenase site behave differently towards $NaBH_4$. Probably, $NaBH_4$ is able to penetrate in the active centre of the enzyme and reduce an essential part of it, possibly a S-S linkage.

Fast and complete photolysis of the rhodopsin in the rod outer segment preparation, in the presence of $NaBH_4$, gave no retinoldehydrogenase inactivation at all, while the regeneration capacity was greatly reduced. As indicated in chapter 4, the most likely explanation of this phenomenon is that retinaldehyde is fastened to the chromophoric site. This does not cause a decrease in retinoldehydrogenase activity. On the contrary, the structural change in the rhodopsin molecule induced by photolysis leads to protection of the active centre of retinoldehydrogenase against $NaBH_4$.

The decrease in retinoldehydrogenase activity and the increase in regeneration capacity, which occur when the time interval between photolysis and reduction is lengthened indicates that the retinaldehyde is bound to an amino group in the active centre of the retinoldehydrogenase. This also points to a gradual migration of retinaldehyde from the chromophoric site to the retinoldehydrogenase site after metarhodopsin II formation. At 5-15 min after illumination, when the retinoldehydrogenase activity has reached its lowest value, maximal occupation of the active centre is reached. The time needed for maximal occupation of the active centre is probably much longer than the time needed in vivo. This discrepancy might very well be due to the experimental conditions,e.g. the absence of the coenzyme. In the absence of coenzyme, retinaldehyde is not reduced to retinol and will be randomly distributed over the amino groups present in the rod outer segment membrane. This leads to a partial restoration of the retinoldehydrogenase activity. The restoration is not complete, probably because the retinaldehyde remains partly bound to the

retinoldehydrogenase site.

Evidence for the migration of the chromophoric retinaldehyde after photolysis is obtained also from entirely different experiments, performed in our laboratory by Drs. W.J.de Grip. When the rod membrane preparation is subjected to four sucessive treatments with methylacetimidate (MAI) in the dark, 40% of the retinoldehydrogenase activity is left, while 92% of the amino groups are blocked. A 5^{th} treatment with MAI completes amidination, leaving 15% retinoldehydrogenase activity if it is carried out in the light and no activity if it is carried out in the dark. In the former case the retinaldehyde can migrate to the enzyme and protect the active site against amidination, while in the latter case no migration of the chromophore occurs and the active site remains unprotected and is apparently amidinated. This is also further proof for the existence of an amino group at the active site.

From our observations of changes in regeneration capacity and retinoldehydrogenase activity in $NaBH_4$-treated and amidinated rod outer segment preparations we conclude that a migration of retinaldehyde from the chromophoric site to the retinoldehydrogenase site occurs during the decay of metarhodopsin II. This means that the enzyme-substrate complex results from a transiminization reaction, in which retinylidene-opsin reacts with the amino group in the active centre. This idea is in accordance with the results of de Pont, Daemen and Bonting (1970[a]), who showed that retinylidene-amines are able to act as substrate for the enzyme.

Under our conditions, the migration of retinaldehyde from the chromophoric site to the retinoldehydrogenase site occurs about as fast (5 min) as the decay of metarhodopsin II to metarhodopsin III in the isolated frog retina (Baumann, 1972). The all-trans retinaldehyde

does not reach the retinoldehydrogenase site before or during metarhodopsin II formation, since it is still bound to the original site immediately after metarhodopsin II formation (chapter 4). This suggests that metarhodopsin III could be the enzyme-substrate complex. At this time we have not yet investigated this possibility.

From rapid measurements of linear dichroism in single frog rods during photolysis, Harosi (unpublished observations, reported at Assoc. for Res. in Vision and Ophthalmol. meeting, Sarasota, 1972) concluded that the chromophore orientation remains parallel to the plane of the membrane through the metarhodopsin II stage, but changes to a nearly perpendicular orientation thereafter. This suggests that the migration might be "channeled" by the adoption of a preferential orientation of the retinaldehyde polyene chain parallel to the fatty acid chains of the phospholipids.

The relative slow migration of the retinaldehyde from the chromophoric site to the retinaldehydrogenase site indicated by the $NaBH_4$ experiments suggests that the enzyme is not part of the opsin molecule. This is also suggested by a consideration of the enzyme activity relative to the number of rhodopsin molecules. The turnover number in our experiments is only 0.5 mole of retinaldehyde per minute per mole of rhodopsin. This activity represents about a quarter of the maximal rate, which indicates a turnover number of approximately 2 per opsin molecule. Since known turnover numbers of various enzymes are upwards from 10.000, this suggests that the enzyme is an entity separate from the opsin molecule occurring less than once per 5.000 opsin molecules. The fact that the enzymatic reaction converts the retinaldehyde molecule with its reactive aldehyde group to a retinol molecule, which is released after its formation, would further explain the "channeled" migration of the chromophores: the enzyme site would act as a "sink" for the chromophore.

CHAPTER 6

FORMATION OF ISORHODOPSIN FROM PHOTOLYZED RHODOPSIN IN DARKNESS

6.1 INTRODUCTION

In order to permit continued function of the rod photoreceptor there must be a continuous resynthesis of rhodopsin from its bleaching products. The mechanism of this regeneration process, which can take place in the dark, is still largely unknown. The crucial step must involve isomerization of the all-trans form of retinaldehyde or retinol to the corresponding 11-cis isomer. It is generally accepted that 11-cis retinaldehyde or retinol is not stored outside the eye (Ames, Swanson and Harris, 1955), but small stores of 11-cis retinol are found in the pigment epithelium (Hubbard and Dowling, 1962; Krinsky, 1958). Hence, there must be a mechanism in the eye which converts all-trans retinaldehyde into the 11-cis isomer. It is also possible that all-trans retinol is isomerized and the resulting 11-cis retinol is converted by oxidation to 11-cis retinaldehyde. The isomerization is an energy-requiring process, as shown by Kropf and Hubbard (1970) from temperature dependence of the isomerization. However, it is not clear which compound or process supplies the energy. Light energy is not directly needed, since regeneration in the light and in darkness occur at the same rate (Rushton, 1957; Lewis, 1957). Possibly the energy is supplied in the form of an energy-rich small molecule like ATP.

The location of the isomerizing system in the eye is not clear. There is experimental evidence that isomerization is possible in the pigment epithelium (Hubbard and Dowling, 1962;

Krinsky, 1958). In the frog eye all-trans retinol, which is the ultimate product of photolysis, can migrate to the pigment epithelium, where isomerization can occur. The isolation of regeneration promoting water-soluble factors from frog pigment layers has been described (Bliss, 1951[b]; Hubbard and Wald, 1951). On the other hand,in the rat the retina itself clearly possesses an isomerizing mechanism (Cone and Brown, 1969; Goldstein, 1970).

We decided to look for an isomerizing mechanism in cattle retina, since we thought that retinaldehyde isomerization within the outer segment could be of considerable physiological importance. The only report of a retinal isomerizing enzyme is that of Hubbard (1956[a]), who reported that an ammoniumsulfate fraction of water-soluble proteins from cattle retina was able to isomerize all-trans retinaldehyde to 11-cis retinaldehyde. This isomerization occurred only when the isomerizing enzyme was present during the illumination of the rhodopsin solution. Since the in vivo rate of regeneration of rhodopsin in rat and man (Lewis, 1957; Rushton, 1957) is equal in darkness and in the light, we investigated whether isomerase activity could be demonstrated in the dark in cattle retina. The activity of the enzyme could be independent of light conditions. The retinal isomerase activity might be associated with a soluble protein, or there might be an isomerase site in the rod sac membrane. It might even form part of the rhodopsin complex, perhaps being located on the same polypeptide chain, which constitutes the chromophore binding site.

In studying the isomerization process the resulting retinaldehyde is made to react immediately to form a photopigment, which is then measured by means of the decrease in 500 nm absorption upon illumination.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of water-soluble retinal protein extract

Forty retinas, suspended in 30 ml physiological saline, were homogenized in a tightly fitting Potter-Elvehjem tube. The homogenate was centrifuged at 30,000 x g for 30 min. The water soluble proteins in the supernatant were precipitated by the addition of ammoniumsulfate to 60% saturation. After solubilization in 5 ml 0.067 M phosphate buffer (pH 7.0) and repeated dialysis against the same buffer, the dialysate was tested for isomerization activity.

6.2.2 Sterilization of rhodopsin preparations

The rhodopsin was chemically sterilized with ethylene oxide. A very concentrated suspension of rhodopsin in 0.067 M phosphate buffer (pH 7.0) was treated in the dark with ethylene oxide (15% in CO_2) at room temperature for 15 hours at a total pressure of 5 kg/cm^2. A control sample was kept at room temperature in normal air. The sterilized rhodopsin sample was left for 3 hours in a nitrogen atmosphere, during which time nearly complete hydrolysis of the excess ethylene oxide occurred.

6.2.3 Growth, isolation and sonication of bacterial cells

Proteus mirabilis was obtained from 300 ml of an overnight culture in brain-heart infusion. The incubation was carried out at 37° without aeration. The bacteria were sedimented by centrifugation (8,000 x g, 30 min). The pellet was repeatedly washed with 0.067 M phosphate buffer (pH 6.5) and resuspended in 30 ml of the same buffer. For incubations of the supernatant from disrupted cells,

2-mercapto-ethanol was added to the bacterial growth medium in a final concentration of 0.05 M. After centrifugation the pellet was resuspended in 10 ml 0.067 M phosphate buffer (pH 6.5), containing 0.05 M 2-mercapto-ethanol. The bacterial suspension was sonicated three times for 30 sec (75 W from a Branson sonifier B-12). During sonication the sample was cooled by an ice-salt mixture, and kept under nitrogen. All subsequent manipulations were also carried out in a nitrogen atmosphere.

6.2.4 Incubation conditions

Photolyzed rhodopsin was prepared by illuminating a rhodopsin suspension (25 µM in 0.067 M phosphate buffer, pH 6.5) for 10 min through orange and infrared filters (OG 370 and KG 1, Schott-Jena) by a 75 W tungsten lamp at 15 cm distance. This leaves about 5-10% of the visual pigment originally present as a mixture of photoregenerated rhodopsin and isorhodopsin. Opsin was prepared as described in 3.2.2. When a mixture of opsin and all-trans retinaldehyde was used in isomerizarion experiments the retinaldehyde was added as a concentrated solution in acetone or methanol and the mixture was preincubated for 30 min, in order to achieve optimal interaction between these two substances. The final solvent concentration in the incubation mixture never exceeded 5%. All incubations with retinal proteins, bacteria and bacterial supernatant were carried out in darkness. The isomerization and recombination reactions were stopped by the addition of 50 µl of a 4:1 mixture of 10% Triton-X-100 and 1,0 M NH_2OH to 250 µl samples from the incubation mixture,

6.2.5 Measurement of the redox potential

The combined Pt-calomel electrode (Radiometer pK 149) was calibrated by measurement of the redox potential of a quinhydrone solution over the pH range 2 to 8. The experimental values were in good accordance with the expected values.

In order to measure the redox potential during incubation of photolyzed rhodopsin with bacteria, the electrode was inserted into the reaction vessel together with a sampling tube and a gas inlet. A mixture of 4 ml 25 µM photolyzed rhodopsin suspension and 1 ml bacterial suspension was incubated at 37^{o} in darkness. The incubation mixture was stirred continuously. The redox potential was recorded continuously, while periodically samples were taken to measure the visual pigment concentration.

After each experiment the electrode was cleaned very carefully with nitric acid.

6.3 RESULTS

6.3.1 Experiments with retinal proteins

When repeating Hubbard's experiments on the retinal isomerase (1956), we observed that in the presence of a water-soluble retinal protein extract a visual pigment was formed in the dark with illuminated rhodopsin (Rotmans, Daemen and Bonting, 1971). Analysis showed that the retinal protein solution contained no retinaldehyde. The synthesis of visual pigment can therefore not be ascribed to a reaction of photolyzed rhodopsin with 11-cis retinaldehyde, but must be due to isomerization of the all-trans retinaldehyde present in the illuminated rhodopsin and subsequent binding of the resulting

isomerized retinaldehyde by opsin.

In the absence of opsin, exogenous all-trans retinaldehyde and all-trans retinol were not isomerized, when incubated with the retinal protein extract.

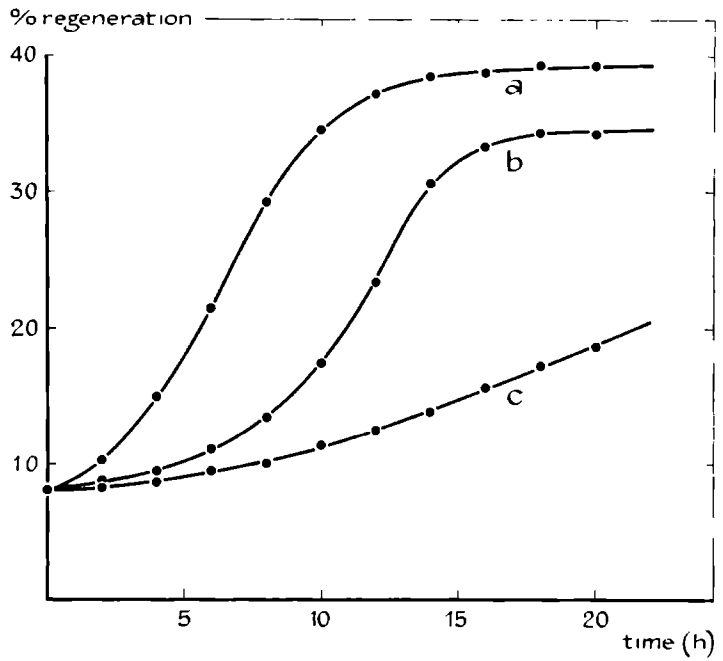

Fig 30. Visual pigment synthesis stimulated by a retinal protein extract. Twenty nmol photolyzed rhodopsin in 1 ml 0.067 M phosphate buffer (pH 6.5) was incubated at room temperature with 300 (a) and 150 (b) µl retinal protein extract in the same buffer. Curve (c) represents the synthesis of visual pigment in a blank containing no retinal protein extract.

In fact, the extract was able to catalyze the reverse reaction; 11-cis retinaldehyde was nearly completely converted to the all-trans isomer. On the other hand, the retinal protein extract was able to stimulate the photopigment formation from all-trans retinaldehyde when opsin was present in the incubation mixture. A certain interaction between all-trans retinaldehyde and opsin or an immediate withdrawal of the isomerized retinaldehyde by opsin seems essential for isomerization of the all-trans isomer.

These results seemed to indicate the presence of an all-trans retinaldehyde isomerase in the retinal protein extract. Closer examination, however, revealed a number of surprising facts.

First, the absorption spectrum of the synthesized photopigment has an absorption maximum at 485 nm, suggesting that it is isorhodopsin, an analogue of rhodopsin not occurring in vivo. This was proven by extracting with 90% ethanol and subjecting the extract to thin layer chromatography. The major spot proved to be 9 cis-retinaldehyde (Rotmans, Bonting and Daemen, 1972).

Secondly, this formation of isorhodopin is rather slow, requiring from 10 to 30 hrs at 20° and 6 to 15 hrs at 37°C to reach its maximum (fig 30). Often a lag-time of several hours was observed. The isorhodopsin yield varied between 30 and 80% of the amount of rhodopsin present, assuming a value of 1.06 for the ratio of the molar absorbance of isorhodopsin and rhodopsin (Hubbard, 1956).

In high concentration photolyzed rhodopsin, upon standing at 20° in the dark in the absence of exogenous all-trans retinaldehyde and retinal protein extract, also yields isorhodopsin (fig 31). Maximal yields (up to 70%) are obtained at rhodopsin concentrations above 30 nmol/ml. The isomerizing retinal proteins apparently merely

accelerate the isorhodopsin formation.

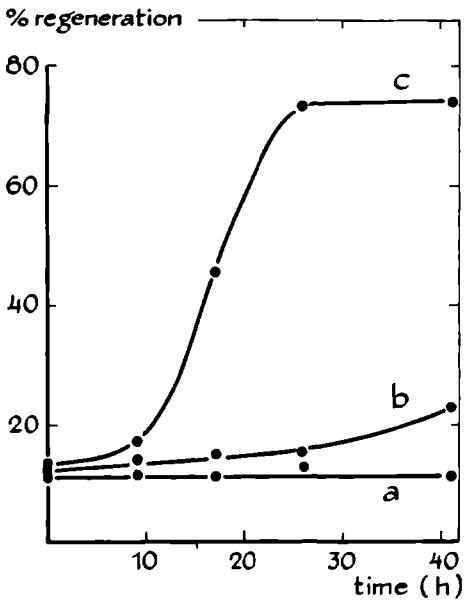

Fig 31. Isorhodopsin formation from photlyzed rhodopsin. Rhodopsin suspended in 0.067 M phosphate buffer was illuminated through orange and infrared filters for 10 min. The illuminated suspension was kept in the dark at room temperature. After certain intervals samples were withdrawn and the amount of isorhodopsin formed was measured and expressed as percentage of the rhodopsin originally present. Three concentrations of rhodopsin were used (a) 4 nmol/ml, (b) 8 nmol/ml, (c) 60 nmol/ml.

Thirdly, the isomerizing activity was retained when the protein extract previously had been exposed to strong acid or base (final pH 1 and 10, respectively) or had been heated to 100° for 5 min. When the retinal protein extract

was subjected to chromatography on Sephadex-G-75, all fractions showed isomerizing activity.

These findings strongly suggest that the isomerizing activity of the extract is not due to an enzyme. The long incubation times required for isomerization and the long time preceding isomerization made us suspect a bacterial involvement.

6.3.2 Role of bacteria in isorhodopsin formation

The possibility of bacterial involvement was studied by the addition of antibiotics to the incubation mixture. Both penicillin and streptomycin (100 mg/ml incubation mixture) inhibited the isomerizing effect of the retinal protein extract completely.

Assuming the bacteria to be present in the rhodopsin suspension, one would expect to obtain stimulation of their growth by preincubating the rhodopsin with the retinal protein extract for several hours at 37° in the dark, where the protein extract would act as a nutrient medium. The resulting suspension, after photolysis, gave indeed a much faster isorhodopsin synthesis than the non-preincubated sample (fig 32). The rhodopsin itself was not affected by the preincubation, since the ΔA_{500} remained unchanged.

Further confirmation for bacterial involvement was obtained by sterilization of the reaction mixture. The rhodopsin was chemically sterilized with ethylene oxide. The retinal protein solution was sterilized by heating at $100^{\circ}C$ for 5 min. Incubations were carried out under sterile conditions. Under totally sterile conditions the formation of photolysable pigment was greatly inhibited (fig 33a), while the use of non-sterile protein extract gave slightly more isorhodopsin (fig 33b), and the normal amount of pigment was formed if both components

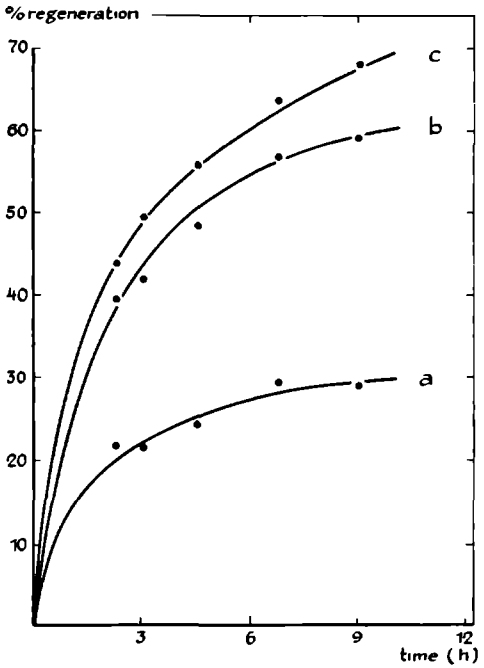

Fig 32. Preincubation of rhodopsin with retinal protein extract. Twenty-five nmol photolyzed rhodopsin in 1 ml 0.067 M phosphate buffer (pH 6.5) is incubated with 1 ml bacterial suspension in the same buffer, at 37°. Curve (a) depicts the isorhodopsin formation during this incubation. In two parallel experiments the rhodopsin suspension, mixed with the bacterial suspension was preincubated for 3 and 6 hrs prior to illumination (curves b and c, respectively).

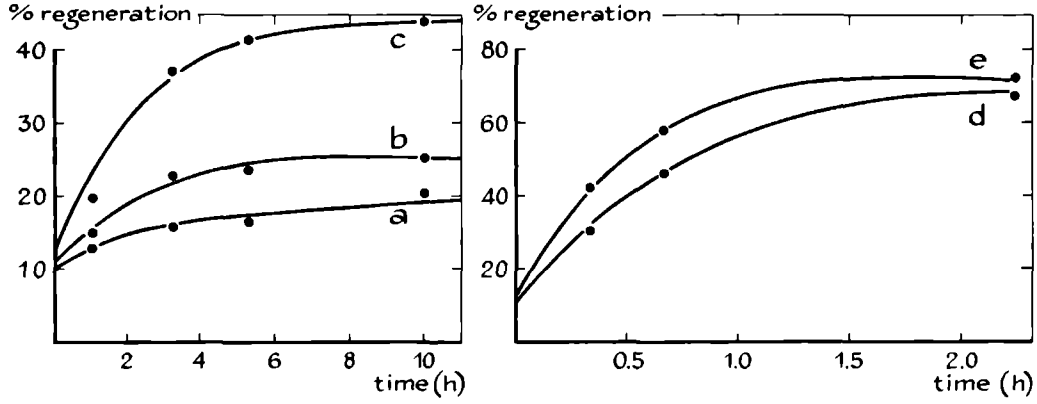

Fig 33. Inhibition of isorhodopsin formation by sterilization with ethylene oxide. Sterilized photolyzed rhodopsin is incubated at room temperature with sterilized retinal protein extract (a), non-sterilized retinal protein extract (b). Curve (c) depicts the isorhodopsin formation from non-sterilized rhodopsin incubated with non-sterilized retinal protein extract. Sterilized photolyzed rhodopsin had retained its capacity to form isorhodopsin when incubated with bacteria (d). Curve (e) represents the course of isorhodopsin formation from non-sterilized photolyzed rhodopsin when incubated with bacteria.

were non-sterile (fig 33c). The ethylene oxide treatment of the rhodopsin preparation did not affect its spectral characteristics or its ability to form isorhodopsin under non-sterile conditions (fig 33d, e). Replacement of the retinal proteins by a typical bacterial growth medium, brain-heart infusion, led to a rapid formation of isorhodopsin in high yields. This indicates that the only function of the retinal protein extract is to serve as a growth medium for the bacteria present in the rhodopsin preparation.

6.3.3 Experiments with washed bacteria

6.3.3.1 Isorhodopsin synthesis from photolyzed rhodopsin

To confirm the bacterial influence in a more direct way, bacteria isolated from an isorhodopsin generating system were cultured overnight at 37° without aeration in 300 ml brain-heart infusion and harvested by centrifugation (8,000 x g, 30 min). The pellet was repeatedly washed with 0.067 M phosphate buffer (pH 6.5) and resuspended in 30 ml of the same buffer. Equal volumes of the bacterial suspension and the illuminated rhodopsin suspension were incubated under nitrogen in the dark at 37°. The amount of isorhodopsin formed reached a maximum after 3 to 4 hours. At that time 65 to 95% of the rhodopsin originally present had been converted to isorhodopsin. Isorhodopsin formation was first order with respect to the opsin concentration and had a $t_{\frac{1}{2}}$ of about 35 minutes (fig 34). Absorption spectra were obtained after solubilization in detergent solution of an aliquot of the original rhodopsin suspension, of the photolyzed suspension and of the suspension after 4 hours incubation with bacteria (fig 35).
Fig 36 shows that the isorhodopsin formed by bacterial action has a normal photolytic behavior.

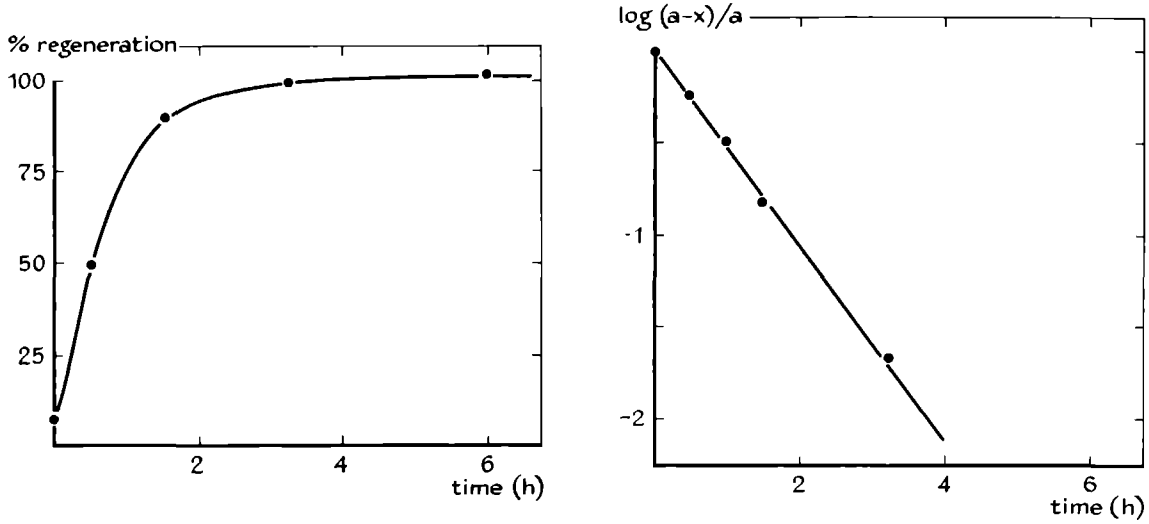

Fig 34. Kinetics of isorhodopsin synthesis by bacterial action. Forty nmol photolyzed rhodopsin in 1 ml 0.067 M phosphate buffer (pH 6.5) was mixed with 1 ml bacterial suspension in the same buffer and incubated at 37° in the dark; 400 µl samples were taken and mixed with 50 µl 1 M NH_2OH and 50 µl Triton-X-100 (10%). In the figure on the left the amount of isorhodopsin, calculated from ΔA_{485}, is expressed as percent of rhodopsin originally present. The figure on the right shows that the isorhodopsin formation is first order for opsin.

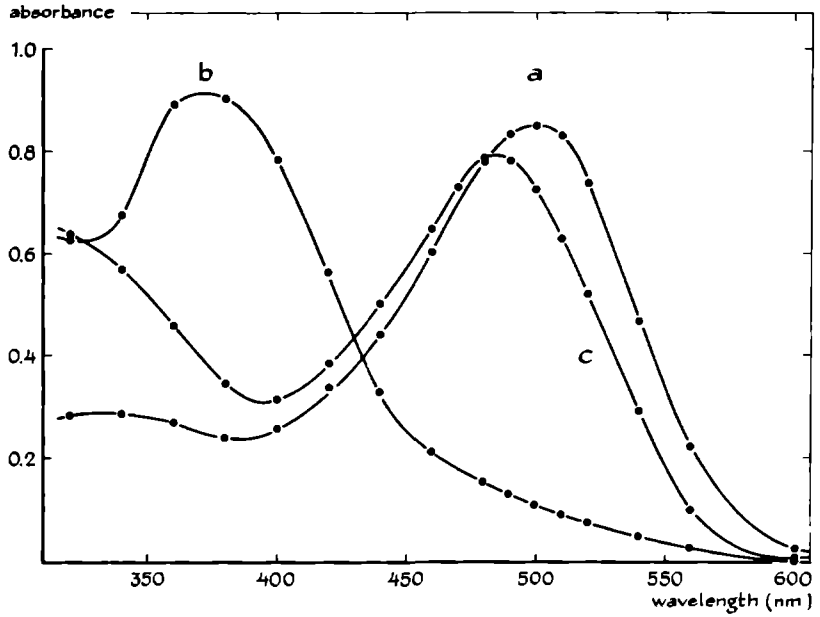

Fig 35. Spectral evidence for the formation of isorhodopsin. Absorption spectra of rhodopsin (a), photolyzed rhodopsin (b) and isorhodopsin formed by bacterial action (c). The pigment suspensions were solubilized in a 1% Triton-X-100 solution in 0.067 M phosphate buffer (pH 6.5) prior to spectral measurement.

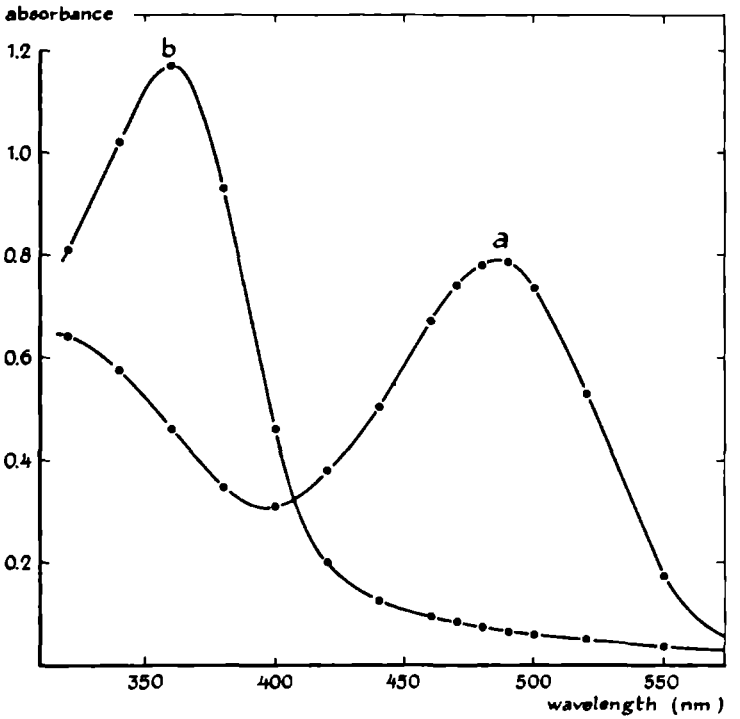

Fig 36. Photolysis of isorhodopsin formed by bacterial action. Absorption spectra are shown for isorhodopsin formed by bacterial action, before (a) and after exhaustive illumination. The pigment was solubilized in a 1% Triton-X-100 solution in 0.067 M phosphate buffer (pH 6.5), containing 0.05 M hydroxylamine.

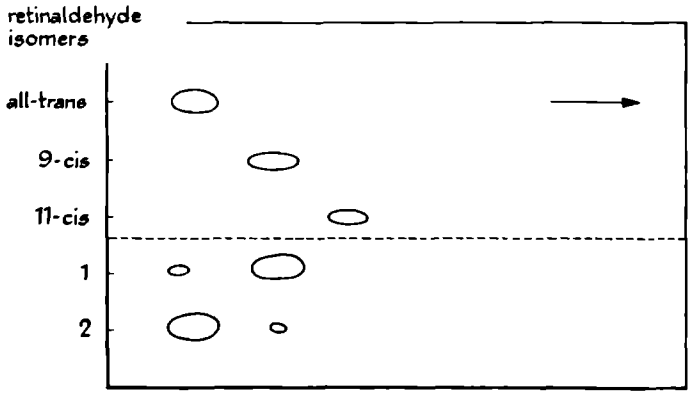

Fig 37. Thin-layer chromatography on silica gel of chromophore extracted, before (1) and after (2) illumination, from isorhodopsin formed by bacterial action. Extraction with 90% ethanol; eluent, hexane-ether (85:15, v/v); detection: spraying with Carr-Price reagent.

Fig 37 demonstrates that 9-cis retinaldehyde is the chromophoric group in the newly formed pigment, whereas all-trans retinaldehyde is the isomer resulting from illumination. The spectral and photolytic properties of rhodopsin and isorhodopsin are apparently not affected by the bacteria. Additions of antibiotics (penicillin or streptomycin, 100 mg/ml incubation mixture) resulted again in complete inhibition of pigment formation. Upon cultivation of the

bacteria isolated from the rhodopsin preparation it was found that several species were present. Rather than isolating everyone of these and testing each species individually for isomerizing activity, we tested a number of organisms available to us in pure cultures. The results indicate that the isomerizing activity is not limited to a single species, although differences with respect to velocity and maximal level were observed (table V). In subsequent experiments the species with the highest isomerizing activity, Proteus mirabilis, a facultative anaerobic organism, was used.

Table V. Maximal regeneration of isorhodopsin by various bacteria

Organism	Maximal regeneration
Proteus mirabilis	77%
Erwina aroidea	55%
Escherichia coli	27%
Arthrobacter	20%
Argo bacterium lumifaciens	18%
Pseudomonas aeruginosa	8%
Bacteria from a non-sterile culture	70%
No bacteria added	8%

All bacteria were cultured in 125 ml brain-heart infusion for 24 hours. After washing and concentration, aliquots of the bacterial suspension were incubated with photolyzed rhodopsin for 6 hours at 37° in the dark.

The bacterial pigment regeneration was dependent on medium pH and incubation temperature. Optimal results were obtained at pH 6.5 (fig 38) and 40° (fig 39).

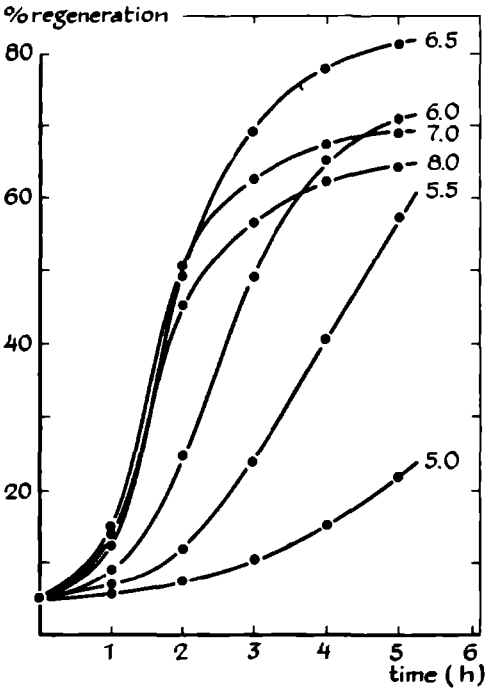

Fig 38. pH dependence of bacterial regeneration. Thirty nmol photolyzed rhodopsin, suspended in 0.067 M phosphate buffer of various pH values is incubated with an equal volume of bacterial suspension in the dark at 37°. The amount of isorhodopsin formed is expressed as percent of the rhodopsin originally present. Incubations were performed at pH 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0.

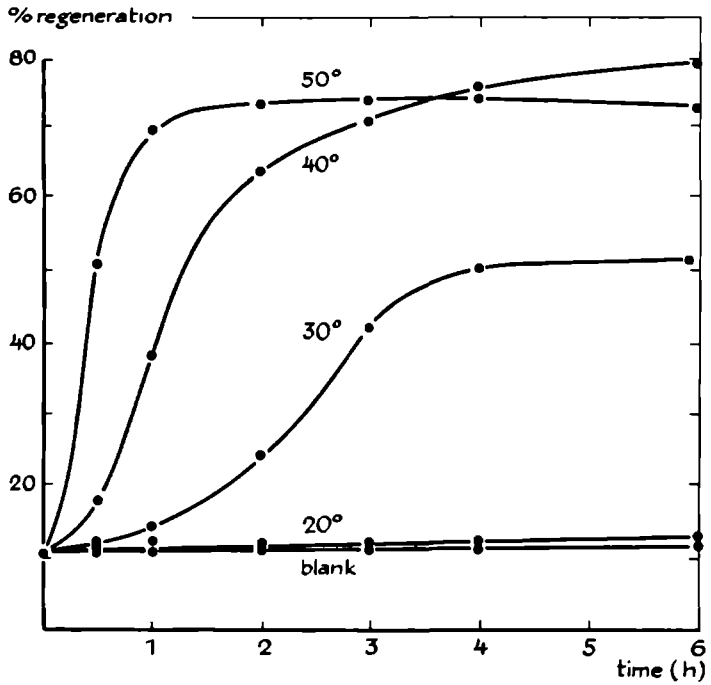

Fig 39. Incubation of photolyzed rhodopsin with bacteria at various temperatures. Twenty nmol photolyzed rhodopsin in 1 ml 0.067 M phosphate buffer (pH 6,5) is incubated with 1 ml bacterial suspension in the dark. Synthesized isorhodopsin is expressed as percent of rhodopsin originally present. Incubations were performed at 20^{o}, 30^{o}, 40^{o} and 50^{o}. The blank (no bacteria present) was equal at all temperatures.

In several experiments the synthesis of isorhodopsin did not start immediately after the addition of the bacteria. The length of this lag phase differed from experiment to experiment. When however the bacterial suspension was preincubated, a lag phase was never observed. The lag phase was also abolished when the bacteria were not washed but immediately incubated with photolyzed rhodopsin. Presumably, the delay in isorhodopsin synthesis is due to metabolic inactivity of the bacteria, Dilution of various bacterial suspensions from different growth phases to the same density, led to the same course of isorhodopsin formation upon incubation with photolyzed rhodopsin. Thus, during logarithmic growth and in the stationary phase, bacteria are equally suitable for regeneration. In all further experiments bacteria were used immediately after harvesting in order to abolish the lag phase.

When no lag phase occurs, it is possible to measure the initial rate of isorhodopsin synthesis. The initial rate is proportional to the concentration of photolyzed rhodopsin (fig 40). The initial rate and the extent of isorhodopsin formation are also dependent on the number of bacteria present at the beginning of the incubation (fig 41). The initial rate is proportional to the logarithm of the bacterial density over a large interval (fig 42).

It is not yet clear how to incorporate these data in the kinetics of the isomerization reaction. The problem is how to define the reaction. Tentatively, we should like to assume that the bacteria produce an isomerizing factor, which acts as a cofactor, while the rod membrane preparation acts as an enzyme, which isomerizes the substrate, all-trans retinaldehyde, and also binds the product, 9-cis retinaldehyde, to form isorhodopsin. The substrate concentration is then known exactly (equal to the original rhodopsin concentration), while the enzyme and cofactor

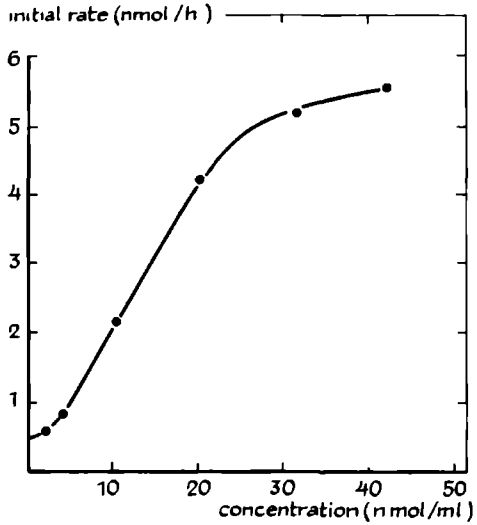

Fig 40. Initial velocity of bacterial regeneration as a function of the concentration of photolyzed rhodopsin. From 2 to 42 nmol photolyzed rhodopsin in 1 ml 0.067 M phosphate buffer (pH 6.5) was incubated at 37° with 25 µl bacterial suspension in the same buffer. No lag-times were observed and the isorhodopsin synthesis was linear over a 140-min period in all experiments.

concentrations may be assumed to be proportional to the photolyzed rhodopsin concentration and the number of bacteria, respectively. The arguments for assuming that the rod membrane preparation acts as an isomerase are provided by the experiments with the bacterial ultrasonic supernatant, described in section 6.3.5.

At very high bacterial densities, the maximal regeneration of visual pigment decreases (fig 41). Retinaldehyde determinations (thiobarbituric acid) in the incubation

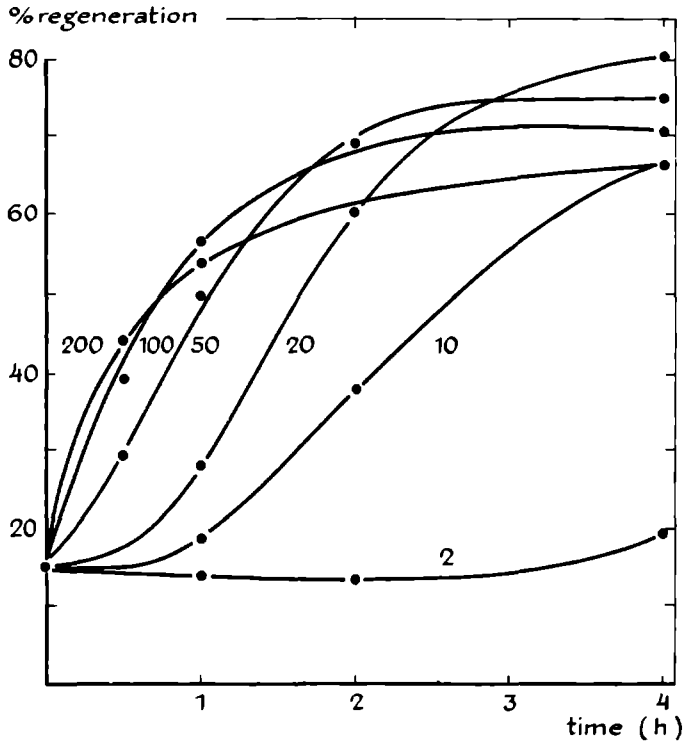

Fig 41. Synthesis of isorhodopsin from photolyzed rhodopsin at various bacterial densities. Incubations were in 0.067 M phosphate buffer pH 6.5 at 37° in the dark. The figures denote the volumes of bacterial suspension in microliters added to 100 µl 30 µM photolyzed rhodopsin. The final volume was 300 µl in all incubations. The amount of isorhodopsin synthesized is expressed as percent of rhodopsin originally present.

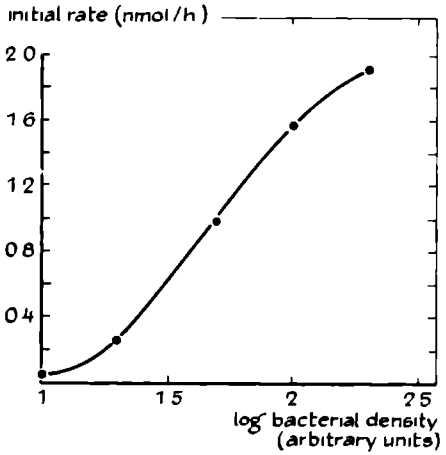

Fig 42. Initial rate of isorhodopsin synthesis as a function of bacterial density. The amount (in nmols) of isorhodopsin synthesized in the first 30 min after addition of bacteria is plotted logarithmically against the number of microliters of bacterial suspension. Experimental conditions as in the previous figure.

mixture after completion of isorhodopsin synthesis showed that, independent of the amount of synthesized isorhodopsin, only bound 9-cis retinaldehyde was present and no excess retinaldehyde. The bacteria must have metabolized part of the all-trans retinaldehyde before complete isomerization of this compound could occur. The simultaneous competitive isomerization and metabolic retinaldehyde conversion explain why isorhodopsin synthesis is always incomplete in our experiments. Only in a few experiments nearly 100% regeneration was observed. In those experiments metabolic degradation of retinaldehyde must have been slow compared to its isomerization. After maximal but incomplete regeneration, the

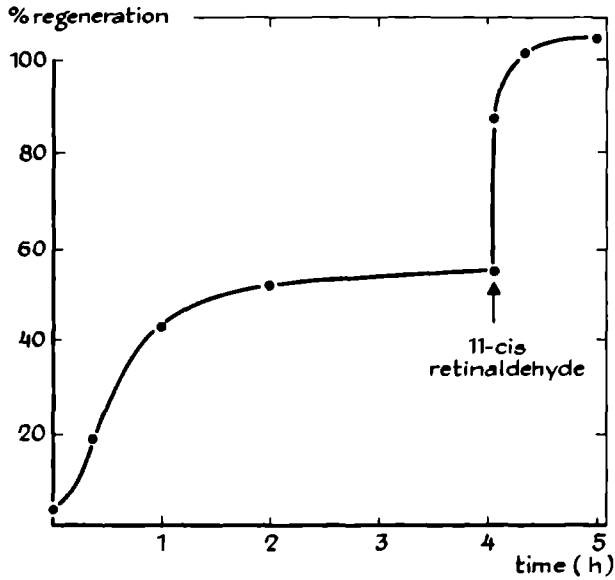

Fig 43. Reaction of photolyzed rhodopsin with 11-cis retinaldehyde after maximal bacterial regeneration. Photolyzed rhodopsin (100 µl of a 40 µM suspension) in 0.067 M phosphate buffer (pH 6.5) is incubated with 100 µl bacterial suspension in the same buffer. Incubation is at 37^{o} in the dark. After 4 h a 10-fold excess of 11-cis retinaldehyde is added. The visual pigment formed is expressed as percent of rhodopsin originally present.

chromophore binding site is still intact, since incubation with 11-cis retinaldehyde leads to complete regeneration (fig 43).

The rate of isorhodopsin formation and the maximal yield did not change during standing for up to 3 hours at 20°; beyond that period both decreased. In digitonin solution the regeneration ability decreased much faster, probably due to more complete release of retinaldehyde and possibly incorporation in separate micelles. Hence, the favorable position of the endogenous retinaldehyde is retained for 3 hours in the absence of digitonin.

6.3.3.2 Role of opsin in isomerization

To see whether opsin is needed for isomerization, we incubated free all-trans retinaldehyde with bacteria for 3 hours at 37°. The incubation mixture was extracted with hexane. The hexane extract was analyzed by thin layer chromatography (silica, eluent: ether-hexane 15/85, v/v) to separate the retinaldehyde isomers. After development, the chromatogram showed no yellow spots. Spraying with Carr-Price reagent showed the presence of only one compound, coloring red rather than blue with the reagent and having the same R_f-value as all-trans retinaldehyde; this is presumably a breakdown product of retinaldehyde. A thiobarbituric acid determination of the retinaldehyde after 3 hours incubation showed that more than 90% had been converted to non-chromogenic products. The breakdown of retinaldehyde was entirely due to bacterial action, since no breakdown occurred when retinaldehyde was incubated for 3 hours at 37° without bacteria. The breakdown of retinaldehyde could be diminished when retinaldehyde-free opsin (NADPH-treated photolyzed rhodopsin, see section 3.2.2) was added. Presumably, the

isomerizing system, which we assume to be present in the opsin preparation, permits isorhodopsin formation to occur in competition with retinaldehyde breakdown. All-trans retinaldehyde added in a 1:1 ratio to retinaldehyde-free opsin reacts for up to 45% to isorhodopsin. The maximal amount of synthesized isorhodopsin during incubation of all-trans retinaldehyde, opsin and bacteria, is proportional to the amount of added all-trans retinaldehyde up to 2 moles per mole opsin. Only half of the added retinaldehyde is used for isorhodopsin synthesis at ratios smaller than 2. The other half is converted by the bacteria to colorless products. At higher ratios, the maximal isorhodopsin synthesis remains at 95% of the amount of opsin initially present (fig 44 and 45). At these higher ratios, the excess retinaldehyde is degraded for more than 90% during the incubation as shown by thiobarbituric acid determinations.

Preincubation of opsin with exogenous all-trans retinaldehyde leads to an increased rate of isorhodopsin synthesis. Apparently the retinaldehyde needs to be bound in some way to the opsin for isomerization. When the incubation of all-trans retinaldehyde with opsin and bacteria is performed in digitonin solution, no synthesis of photopigment is observed. Probably separate micelles of opsin and retinaldehyde are formed, so that no appropiate interaction occurs. Furthermore digitonin might cause a decreased bacterial growth.

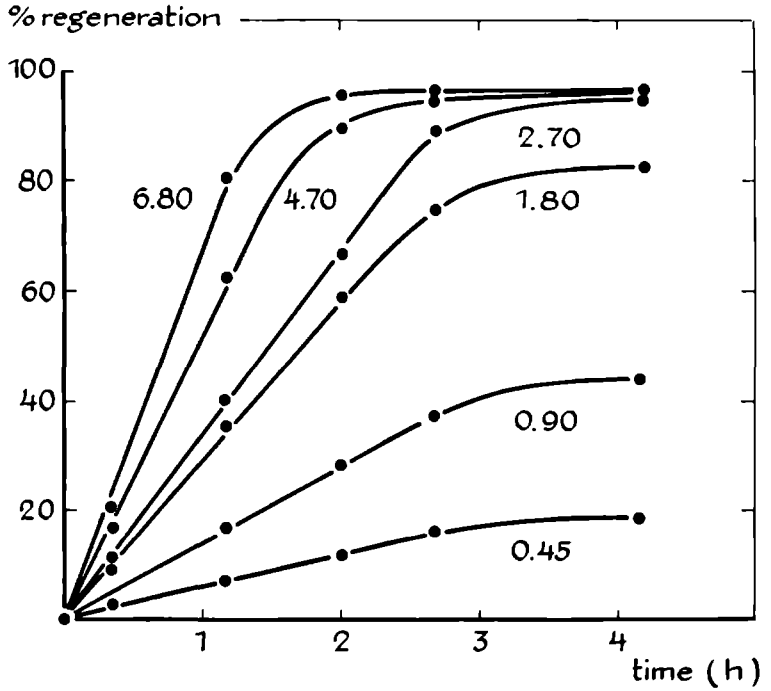

Fig 44. Isorhodopsin synthesis from retinaldehyde-free opsin and all-trans retinaldehyde. Opsin, suspended in 0.067 M phosphate buffer pH 6.5, was preincubated at 37°, in the dark, with various amounts of all-trans retinaldehyde. After addition of bacteria, the isorhodopsin synthesis was followed. The initial opsin concentration was 25 nmol/ml.

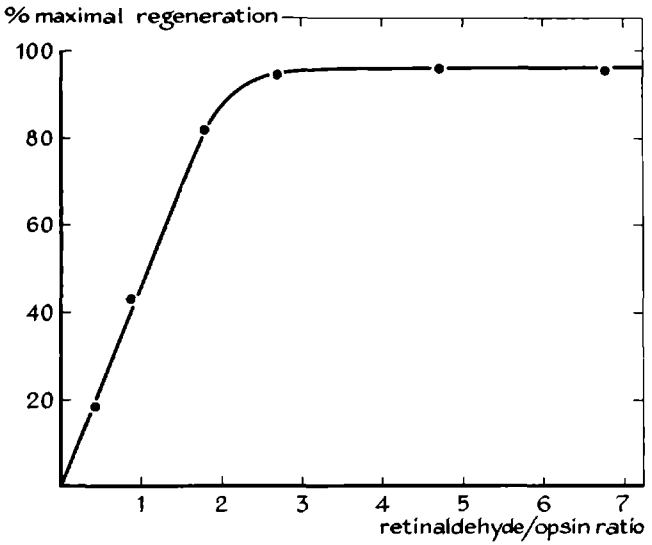

Fig 45. Maximal synthesis of isorhodopsin (expressed as percent of opsin initially present) as a function of the retinaldehyde/opsin ratio.
(initial opsin concentration: 25 nmol/ml.)

6.3.4 Redox potential and isorhodopsin formation

6.3.4.1 Introduction

The redox potential is the voltage difference between an inert metal electrode and a reference electrode, when placed in a solution containing a reversible redox system. In our experiments a platinum electrode and a calomel electrode were used.

If the redox potential of a bacterial nutrient solution is measured and this solution is then inoculated with bacteria, the redox potential changes to more negative values. This change is due to the uptake of oxygen by the growing bacteria. The rate and extent to which the redox potential becomes more negative are dependent on the aeration, the growth rate, the growth phase and the type of bacteria. Aerobic, facultative anaerobic and anaerobic bacteria reach increasingly negative redox potentials. It is rather difficult to interpret the redox potential of a bacterial culture medium. Several redox systems are involved, of which only the oxygen system is known. Moreover, not all redox reactions are necessarily reversible and an equilibrium state is not obtained.

A further difficulty in the application of redox measurements is that the bacteria themselves cannot register a potential at the electrode. The measured redox potential reflects the redox state of the medium, which need not to be in equilibrium with that of the cell itself. However, there is a clear correlation between the redox potential of the medium and the redox state of various compounds (e.g. cytochromes) inside the cell (Baumberger, 1939). The redox state of the medium is probably determined by the excretion of bacterial products with redox character and by the variation in oxygen pressure resulting from oxygen uptake by the

micro-organism (Jacob, 1970). When the redox potential has reached a constant negative value, it is determined by the rate of production of reduced compounds by the cell and the rate of oxidation of these compounds by oxygen, diffused into the culture. The nature of the reduced compounds generally is not known.

6.3.4.2 Results

It was not possible to measure the redox potential in a sample withdrawn from the culture, because during sampling oxygen enters the fluid. Even the potential of the total incubation mixture increases when samples are taken. Therefore a sampling tube was introduced, which could take samples without changing the nitrogen atmosphere above the incubation mixture. In this case no change in redox potential in the mixture was observed. When the electrode was inserted into the incubation mixture, it takes some time before a constant redox potential value is obtained. Hence, continuous measurement of the redox potential was preferred.

Immediately after the start of the incubation of Proteus mirabilis with photolyzed rhodopsin, the redox potential rapidly became more negative, until a value of about -100 mV was reached. Isorhodopsin synthesis would commence only at this low redox potential. But the low redox potential as such was not sufficient, since isorhodopsin synthesis did not necessarily start under these conditions. Hence, the reducing nature of the incubation mixture alone was not sufficient for the isomerization process to start. This was surported by the observation that artificial lowering of the redox potential by the addition of strong reductants (sodiumdithionite, hydroquinone or dithiothreitol) to the medium did not initiate isorhodopsin formation.

For the initiation of isorhodopsin synthesis, the incubation medium must contain a bacterial reductant, which can only exist at a low redox potential.

After the start of isorhodopsin synthesis, the redox potential of the incubate changed to more positive values, parallel to the isorhodopsin synthesis. This change might be due to alteration of the electrode surface, but also to the utilization of reduced substances by the isorhodopsin generating system. The latter possibility seems more likely, since a culture without photolyzed rhodopsin showed no increase in redox potential.

The bacterial isomerization process could easily be inhibited by the addition of oxygen. Even a small addition caused the redox potential immediately to become positive (fig 46). In the incubation medium the concentration of redox potential determining substances, necessary for isorhodopsin synthesis, must be very low, since the redox buffer capacity of the medium was very low. Immediately after the administration of oxygen the isorhodopsin synthesis stopped. The restoration of the redox potential, which occurred after oxygen administration was stopped, was not sufficient for the start of the isorhodopsin synthesis. For that purpose the incubation mixture must be sealed from the air or flushed with nitrogen. The introduction of a nitrogen atmosphere above the incubation mixture also leads to a resumption of isorhodopsin synthesis.

6.3.5 Experiments with supernatants of ultrasonically disrupted cells

Ultrasonic treatment of a concentrated bacterial suspension usually caused complete disappearance of the isomerizing activity. However, when ultrasonication was

performed in a nitrogen atmosphere in the presence of 0.05 M 2-mercapto-ethanol, most of the activity was retained.

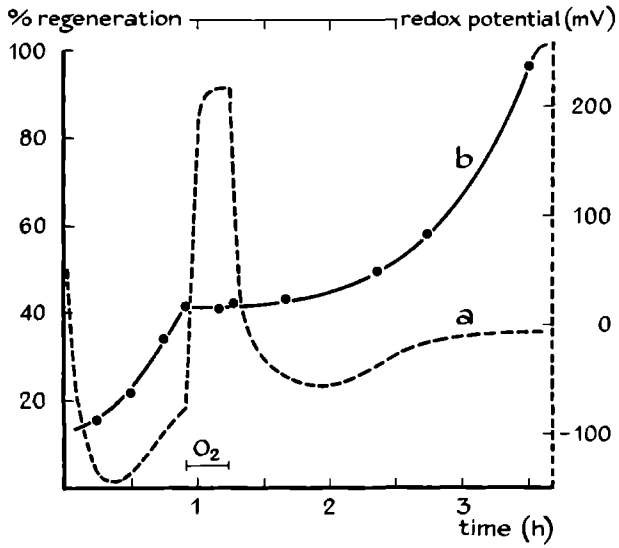

Fig 46. Inhibition of isorhodopsin synthesis by oxygen. Photolyzed rhodopsin (25 nmol) in 1 ml 0.067 M phosphate buffer (pH 6.5) was incubated with 300 µl bacterial suspension in the same buffer. The incubation was performed under a nitrogen atmosphere in the dark at 37°. From the 55th to the 75th min oxygen was administered. The redox potential was continuously recorded (curve a), while the regeneration percentage was determined periodically (curve b).

Incubation of photolyzed rhodopsin at 37° with supernatant

obtained by centrifugation (125,000 x g, 30 min) yielded isorhodopsin. A maximal regeneration of 50-80% was reached in 2-3 hours (fig 47).

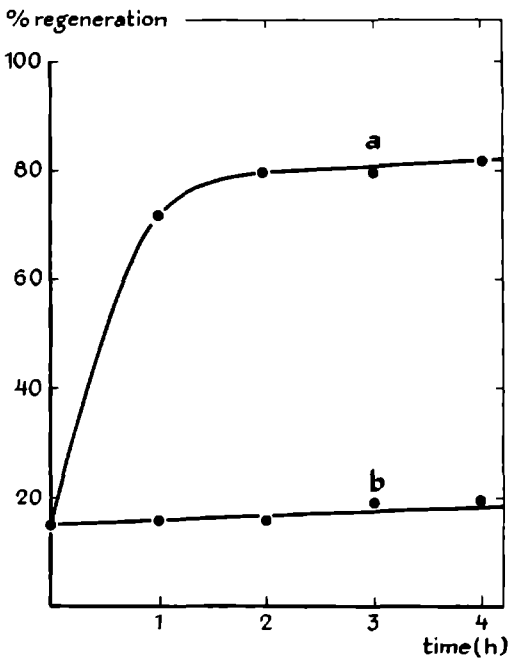

Fig 47. Isorhodopsin formation from photolyzed rhodopsin, catalyzed by bacterial supernatant. Photolyzed rhodopsin suspended in 300 µl 0.067 M phosphate buffer pH 6.5 is incubated at 37° with 400 µl bacterial supernatant. The initial concentration of photolyzed rhodopsin is 25 nmol per ml incubate. The amount of isorhodopsin synthesized is expressed as percent of photolyzed rhodopsin originally present (a). Curve (b) shows that nearly no isorhodopsin is synthesized in the absence of bacterial supernatant.

A control experiment, in which 0.05 M 2-mercapto-ethanol instead of the bacterial supernatant was added, showed no regeneration, indicating that this thiol compound has no isomerizing effect. The isomerizing activity was not caused by a stimulation of new bacterial growth in the rhodopsin suspension by the proteins of the bacterial homogenate or supernatant. Rather it is a distinct compound in the bacterial homogenate or supernatant itself which caused isorhodopsin synthesis, because the synthesis started always without a lag phase and proceeded much faster than after addition of retinal proteins. Moreover, the activity of the bacterial supernatant was not inhibited by penicillin or streptomycin.

Determination of retinaldehyde during the incubation showed that the amount of retinaldehyde remained constant during the whole incubation. Thus, the bacterial supernatant does not cause degradation of free all-trans retinaldehyde as was observed upon incubation with whole bacteria. No detectable formation of 9-cis retinaldehyde was obtained when free all-trans retinaldehyde was incubated with bacterial supernatant, while addition of opsin immediately gave rapid formation of isorhodopsin.

The rate of isorhodopsin synthesis increased with the amount of supernatant added. The concentration of the active compound in the supernatant differed in each preparation, since the amount of supernatant giving maximal pigment synthesis, varied considerably. Maximal synthesis after incubation with an optimal amount of supernatant varied between 50 and 80% of the photolyzed rhodopsin initially present. The maximal regeneration after incubation with bacterial supernatant was usually lower than obtained after incubation with intact bacteria. Neither the rate nor the maximal regeneration could be increased by addition of a second aliquot of supernatant

after 1 hour incubation, indicating that the initial amount of supernatant added was sufficient to give maximal regeneration.

The activity of the bacterial homogenate and supernatant was completely and irreversibly destroyed by oxygen and by low concentration of hydrogen peroxide. The stability of the active factor in the bacterial supernatant is preserved best in the presence of 0.01 M 2-mercapto-ethanol. The 2-mercapto-ethanol itself does not stimulate the isorhodopsin synthesis.

We tried to purify the active compound by chromatography of the supernatant on Sephadex-G-50. No isomerizing activity was found in the fractions containing the large proteins, which are eluted with the void volume. The activity was located in the fractions containing low molecular weight compounds. Nearly all proteins could be removed from the bacterial supernatant by freeze-thawing without affecting the isomerizing activity. These observations suggest that the active factor is not a protein. After heating the bacterial supernatant still possesses isomerizing activity. Hence, the activity appears to be due to a relatively small heat stable non-protein molecule. We have not yet succeeded in identifying the active compound, since the investigations are greatly impeded by the oxygen sensitivity of this compound.

6.4 DISCUSSION

We were not able to confirm the results of Hubbard (1956[a]) on the presence of a retinaldehyde isomerase in a soluble retinal protein extract. The existence of this enzyme seems highly doubtful, since two other laboratories have also been unsuccessful in repeating her experiments (Plante and Rabinovitch, 1972; Amer and Akhtar, 1972[b]).

The stimulation of isorhodopsin synthesis from photo-

lyzed rhodopsin by a retinal protein extract in our experiments is merely due to its bacterial growth promoting effect. The observations decribed in this chapter demonstrate that experiments on the regeneration of rhodopsin are easily obscured by the action of bacteria. High concentrations of rhodopsin, the presence of non-rhodopsin protein, and an incubation temperature at 37^{o} are conditions, which lead to a considerable growth of bacteria. Action of these bacteria on illuminated rod outer segments causes a rapid isomerization of all-trans retinaldehyde to 9-cis retinaldehyde, followed by synthesis of isorhodopsin.

The reason for the formation of 9-cis retinaldehyde instead of the 11-cis isomer is not clear. One possibility is that some high-energy compound or coenzyme is lost during the isolation procedure. We therefore tested the effect of adding ATP, NAD^{+} or $NADP^{+}$ (4-fold molar excess to photolyzed rhodopsin), but we did not obtain a detectable formation of rhodopsin.

Tentatively we assume that the bacteria form an isomerizing factor, which acts as a cofactor, while the rod membrane preparation acts as an enzyme, which isomerizes the substrate, all-trans retinaldehyde, and also binds the product, 9-cis retinaldehyde, to form isorhodopsin. The involvement of an isomerizing site on the rod sac membrane seems likely, since neither isomerization nor destruction of all-trans retinaldehyde was detected upon incubation with bacterial supernatant alone, while addition of the rod outer segment preparation caused rapid isorhodopsin synthesis. However, we cannot completely exclude the possibility that withdrawal of small amounts of 9-cis retinaldehyde by opsin might shift the isomerization equilibrium and thereby stimulate isorhodopsin synthesis. If we assume that there is an isomerizing site on the rod outer segment membrane, then this site is probably located

on the opsin molecule, since photolyzed rhodopsin, which has a 1:1 retinaldehyde/opsin ratio, is a more suitable substrate for isomerization than a 1:1 mixture of all-trans retinaldehyde and opsin. The former substrate gives up to 95% isorhodopsin, and the latter only 45%. Presumably the all-trans retinaldehyde in photolyzed rhodopsin is in a more favorable position for isomerization than the exogenous all-trans retinaldehyde.

For a further explanation of our findings three observations are important. First, the synthesis of isorhodopsin occurs in darkness, so a light-activated isomerization of the chromophoric group is excluded. Secondly the bacterial system only works in the presence of opsin, but not on free retinaldehyde alone. This indicates a role of opsin in the isomerization process. Thirdly, the sensitivity of the pigment formation towards oxygen suggests that the substance produced by the bacteria is easily oxidized.

Futterman, Rollins and Vacano (1970) have reported complete regeneration of photopigment in 90 min, when a concentrated suspension of photolyzed rhodopsin (55 nmol/ml) is incubated with a seven-fold molar amount of all-trans retinaldehyde. While the published paper refers to the pigment as "isorhodopsin" Dr Futterman has recently reported to us privately that the pigment is actually isorhodopsin. The incubation had to be carried out in the dark at 37^{o} under anaerobic conditions. A high concentration of the reducing agent dithiothreitol (DTT) causes regeneration to occur also under aerobic conditions (Zorn and Futterman, 1971). When repeating these regeneration experiments, we obtained very erratic results. We found up to 60% regeneration of isorhodopsin in 2 h(without DTT : 25%). We could not find any isomerization of free all-trans retinaldehyde catalyzed by DTT. The experimental conditions and the synthesis of isorhodopsin instead of rhodopsin suggest that they also had a regeneration by bacterial action. We do not wish to

claim, however, that the isorhodopsin formation in this type of experiments is solely due to the bacterial action. The fast and complete synthesis of isorhodopsin from photolyzed rhodopsin and catalytic amounts of reduced riboflavin, as reported by Futterman and Rollins (1971) is probably not disturbed by bacterial action. Whether or not there is a relationship between dihydroriboflavin-catalyzed regeneration and regeneration by bacterial action is not clear. The bacterial supernatant very likely contains trace amounts of riboflavin. However, the primary initiators of retinaldehyde isomerization in Futterman's and our experiments might be different. This need not necessarily mean that also the actual mechanism is different. Both isomerizations are from the all-trans isomer to the 9-cis isomer and both occur in strongly reducing medium.

The vertebrate eye contains no large amounts of riboflavin as found in the eyes of some fishes (Euler and Adler, 1934; Wald, 1935). In these fishes, however, only the pigment epithelium is rich in riboflavin (500 γ per g wet weight) and not the retina. Moreover, the amounts of riboflavin vary considerably in different fish species.

Recently Amer and Akhtar (1972[a]) have reported a complete resynthesis of visual pigment upon dark-incubation of photolyzed rod outer segments with an equivalent quantity of all-trans retinaldehyde for 4 hrs. In a second paper, the isolation of a water-soluble retinal protein with isomerizing activity was reported (Amer and Akhtar, 1972[b]). It was isolated by freeze-thawing of a rod outer segment suspension, followed by centrifugation. The rhodopsin containing pellet had lost its regenerating capacity, but it could be restored by addition of the supernatant. The activity of the supernatant is lost upon heating at 100^{o} for 5 min and is associated with a high molecular weight component.

The very high concentration of rhodopsin (110 nmol/ml),

the presence of non-rhodopsin material, and the incubation temperature of 37° are conditions which favor a considerable growth of bacteria. Action of these bacteria on photolyzed rod outer segments probably has caused a rapid synthesis of isorhodopsin. This synthesis is speeded up by the presence of exogenous all-trans retinaldehyde. The data provided by Amer and Akhtar indeed indicate that bacterial isorhodopsin synthesis has occurred. First, the spectrum of the photopigment formed shows a peak at about 485 nm, which is the wavelength of maximal absorption of isorhodopsin. The occurrence of bacterial isorhodopsin synthesis is further indicated by the inhibition of the regeneration process by digitonin, formaldehyde and lower temperature (20°).

A recent observation by Dr Dartnall, which he communicated to us, suggests that isorhodopsin formation can occur postmortem. He extracted visual pigment from the eye of a Giant Panda, which had died in the London Zoo. He found, in addition to rhodopsin (λ_m = 495 nm), the presence of about 30% of a pigment with λ_m = 487 nm. When he carried out an experiment with an ox eye under similar pre-assay conditions he also observed the presence of some 490 nm pigment. He concluded that in both cases postmortem formation of a non-native isorhodopsin, presumably by bacterial influence, must have occurred.

It is, however, very unlikely that bacterial formation of isorhodopsin could occur in vivo. Yet, an analogy to the rhodopsin regeneration in vivo cannot be excluded, since in both processes isomerization of all-trans retinaldehyde can occur in darkness. The actual role of opsin in the isomerization is not yet completely understood. It may contain isomerase activity, or it may shift the equilibrium between retinaldehyde isomers by withdrawing 9-cis retinaldehyde to form isorhodopsin. The isorhodopsin formation could be of considerable importance for the understanding of the physiological regeneration process, if opsin is

directly involved in the isomerization reaction. The analogy with the in vivo regeneration would be much less clear, if the opsin function would consist only of the withdrawal of 9-cis retinaldehyde from the isomerization equilibrium.

SUMMARY

The main point of studies of the visual process has been the problem of excitation. Much less attention has been paid to the regeneration of rhodopsin, which process must occur to permit continuous functioning of the eye. In this connection the reactions of the chromophoric retinaldehyde are of main interest. Three possible reactions of retinaldehyde have to be considered. First transiminization, which is very well possible since an excess of amino groups is present in the rod outer segment preparation. Secondly enzymatic reduction to retinol, and thirdly isomerization which inevitably has to occur to permit the necessary rhodopsin resynthesis.

In chapter 1, our current knowledge of the morphological and chemical characteristics of the vertebrate visual system is surveyed. Special attention has been paid to visual pigment regeneration. In this connection the question whether reisomerization occurs in the retina itself (short cycle) or in the pigment epithelium (long cycle) has been discussed.

The only isomer of retinaldehyde, which reacts with opsin under formation of rhodopsin, is 11-cis retinaldehyde (Hubbard and Wald, 1952). However, this does not prove that 11-cis retinaldehyde is present as such in rhodopsin. In chapter 2 the extraction of chromophoric retinaldehyde with 90% ethanol, and the analysis of this extract by thin-layer chromatography and iodine-catalyzed photoisomerization is described. The presence of 11-cis retinaldehyde in the hexane extract directly proves the occurrence of the isomer in native rhodopsin. Since one mole of rhodopsin contains one mole retinaldehyde, it was possible to calculate the molar absorbance coefficient by comparing the absorbance of rhodopsin in detergent solution with the absorbance of its extracted chromophore in hexane solution.

In chapter 3 a study of the reaction between photolyzed rhodopsin and 11-cis retinaldehyde is presented. In connection with the work described in chapters 4 and 5, we investigated whether resynthesis of rhodopsin from photolyzed pigment and 11-cis retinaldehyde takes place in suspensions of rod outer segment membranes, that is without solubilization of the reactants in detergent. We could show that such a suspension in phosphate buffer regenerates rhodopsin after addition of 11-cis retinaldehyde to the same extent as after solubilization in digitonin. We further showed that the incomplete recombination at 1:1 retinaldehyde/opsin ratio due to non-specific isomerization of part of the added 11-cis retinaldehyde to all-trans isomer, which does not give any photopigment formation. We also showed that in suspensions 60% of the all-trans retinaldehyde formed upon photolysis, remains covalently bound to amino groups of the opsin molecule. The question whether all-trans retinaldehyde remains bound to the original amino group or migrates to other amino groups was answered by the experiments described in chapter 4.

To determine whether transiminization occurs, we "fixed" the chromophoric retinaldehyde to its binding site by treatment with $NaBH_4$ during or after illumination and then "probed" the original chromophore binding site by incubation with 11-cis retinaldehyde. Metarhodopsin II is the first photolytic product which can be reduced by $NaBH_4$. Hence, reduced metarhodopsin II is formed, when a rhodopsin suspension is illuminated in the presence of $NaBH_4$. The reduced metarhodopsin II gives no photopigment after incubation with 11-cis retinaldehyde, indicating that no chromophore migration takes place during photolysis. Upon enlargement of the time span between photolysis and reduction, formation of retinylrhodopsin took place in increasing amounts. This implies a gradual migration of retinaldehyde after photolysis, rendering the chromophoric

site available to 11-cis retinaldehyde.

In chapter 5 we showed that the active site of the enzyme retinoldehydrogenase is the "receiving site" for the all-trans retinaldehyde. We first compared the properties of the enzyme solubilized in detergents with those obtained from enzyme suspensions in phosphate buffer. The dependence of enzymatic conversion and accumulation of substrate (all-trans retinaldehyde) in the membrane particles indicate that in suspension the configuration around the active centre remains better intact than after solubilization in detergents. The stability of the enzyme in suspension appeared to be much higher than in detergents. Moreover, the maximal conversion rate measured in suspension was about twice as high as in Triton-X-100, even when the measurement was done immediately after solubilization.

Illumination of a rod membrane suspension in the presence of $NaBH_4$ gave no decrease in retinoldehydrogenase activity, indicating that in reduced metarhodopsin II the active centre of the enzyme is not blocked by the retinyl group. However, there is a decrease in retinoldehydrogenase activity, when the time span between photolysis and reduction is enlarged. This indicates that during the decay of metarhodopsin II, retinaldehyde migrates from its original binding site to the active centre of the retinoldehydrogenase, where it is linked to an amino group.

In chapter 6 efforts to detect a retinaldehyde isomerase are described. Initially it seemed that we had found such an enzyme, but later experiments showed that bacteria present in the incubation mixture produced a compound catalyzing the isomerization of all-trans retinaldehyde to the 9-cis isomer in darkness. Photolyzed rhodopsin incubated with intact bacteria of different strains as well as supernatant of an ultrasonicate of bacteria, showed rapid isorhodopsin synthesis. The isomerization occurs only when opsin is present in the incubation mixture. The precise role of opsin in the isomerization process and the identity of the bacterial factor are not yet known.

SAMENVATTING

De onderzoekingen naar het mechanisme van het visuele proces hebben voornamelijk betrekking op het probleem van excitatie. Veel minder aandacht werd geschonken aan de rhodopsine regeneratie, het proces dat onafgebroken functioneren van het oog mogelijk moet maken. In dit verband zijn de reacties van het chrofore retinaldehyde van groot belang. Drie mogelijke reacties van het retinaldehyde zijn in dit proefschrift onderzocht. Ten eerste, transiminisatie; een reactie die gemakkelijk optreedt omdat een overmaat aminogroepen in het staafjes buiten segment preparaat aanwezig is. Ten tweede, enzymatische reductie tot retinol, en ten derde isomerisatie, zonder welke reactie de noodzakelijke rhodopsine resynthese niet kan plaats vinden.

In hoofdstuk 1 is een overzicht gegeven van onze huidige morfologische en chemische kennis van het visuele systeem van de vertebraten, waarbij aan de regeneratie van het visuele pigment bijzondere aandacht is besteed. In dit verband is ook de vraag behandeld of regeneratie in de retina plaats vindt (korte cyclus) of in het pigment epiteel (lange cyclus).

11-cis retinaldehyde is het enige retinaldehyde isomeer, dat met opsine tot rhodopsine reageert (Hubbard en Wald, 1952). Dit bewijst echter niet dat 11-cis retinaldehyde als zodanig in rhodopsine aanwezig is. In hoofdstuk 2 is de extractie van chromofoor retinaldehyde met 90% ethanol beschreven. Door middel van dunne laag chromatografie en jodium-gekatalyseerde fotoisomerisatie werd aangetoond dat het extract voornamelijk het 11-cis isomeer van retinaldehyde bevatte. Dit is een direct bewijs voor het voorkomen van dit 11-cis isomeer in native rhodopsine. Aangezien de molaire verhouding rhodopsine/retinaldehyde één is, is

het mogelijk om de molaire extinctie coefficient van rhodopsine te berekenen door de extinctie van rhodopsine te vergelijken met de extinctie van hieruit geëxtraheerd retinaldehyde.

Hoofdstuk 3 handelt over de reactie tussen belicht rhodopsine en 11-cis retinaldehyde. In verband met de in de hoofdstukken 4 en 5 beschreven experimenten, is onderzocht of rhodopsine resynthese, uitgaande van belicht pigment en 11-cis retinaldehyde, in suspensie plaats kan hebben, dus zonder de beide componenten in detergens op te lossen. Aangetoond werd dat de maximale rhodopsine regeneratie in digitonine en in suspensie gelijk is. De sub-maximale rhodopsine resynthese bij een retinaldehyde/opsine verhouding van één, bleek het gevolg van a-specifieke isomerisatie van een gedeelte van het toegevoegde 11-cis retinaldehyde tot het all-trans isomeer, dat geen vorming van photopigment geeft. Verder werd gevonden dat na belichting in suspensie 60% van het gevormde all-trans retinaldehyde, covalent gebonden blijft aan aminogroepen van het opsine. De kwestie of all-trans retinaldehyde gebonden blijft aan de oorspronkelijke aminogroep of migreert naar andere aminogroepen werd opgelost door de experimenten die beschreven zijn in hoofdstuk 4.

Om te bepalen of transiminisatie optreedt, werd het chromofore retinaldehyde op de bindingsplaats gefixeerd door behandeling met $NaBH_4$ gedurende of na belichting. De mogelijkheid van de oorspronkelijke bindingsplaats van het chromofoor om met 11-cis retinaldehyde te reageren, werd onderzocht. Metarhodopsine II is het eerste fotolyse product dat door $NaBH_4$ gereduceerd kan worden. Daarom wordt gereduceerd metarhodopsine II gevormd, als een rhodopsine suspensie belicht wordt na toevoeging van $NaBH_4$. Het gereduceerde metarhodopsine II vormt geen fotopigment na incubatie met 11-cis retinaldehyde. Dit wijst erop dat

er gedurende de fotolyse geen migratie van de chromofore groep plaats heeft. Bij verlenging van de tijdsduur tussen belichting en reductie, vindt een toenemende synthese van retinylrhodopsine plaats. Dit betekent dat na belichting een geleidelijke migratie van retinaldehyde leidt tot beschikbaar komen van de chromofore bindingsplaats voor 11-cis retinaldehyde.

In hoofstuk 5 wordt aangetoond dat de"ontvangende plaats" voor het all-trans retinaldehyde, het actieve centrum van het enzym retinoldehydrogenase is. Allereerst worden de eigenschappen van het enzym, opgelost in detergentia vergeleken met die verkregen met behulp van enzym suspensies. De pH afhankelijkheid van de enzymatische omzetting duidt erop dat in suspensie de configuratie rondom het actieve centrum beter intact blijft dan in detergentia. De stabiliteit van het enzym in suspensie bleek veel groter dan in detergentia. Bovendien was de maximale omzettingssnelheid, gemeten in suspensie ongeveer tweemaal zo groot als in Triton-X-100.

Belichting van een staafjes buiten segemnt suspensie in aanwezigheid van $NaBH_4$, gaf geen daling van de retinoldehydrogenase activiteit. Dit geeft een aanwijzing dat in gereduceerd metarhodopsine II het actieve centrum van het enzym niet door een retinylgroep geblokkeerd. Echter, een daling in retinoldehydrogenase activiteit treedt op, bij verlenging van de tijdsduur tussen belichting en reductie. Dit wijst op een migratie van retinaldehyde van de oorspronkelijke bindingsplaats naar het actieve centrum van het retinoldehydrogenase, gedurende ontleding van metarhodopsine II.

In hoofdstuk 6 worden pogingen beschreven om een retinaldehyde isomerase aan te tonen. Aanvankelijk leek het dat we een dergelijk enzym geisoleerd hadden; echter latere experimenten toonden aan dat bacterien, aanwezig in het

incubatie medium, een component produceren die in het donker de isomerisatie van all-trans retinaldehyde naar het 9-cis isomeer katalyseert. Belicht rhodopsine geincubeerd met zowel intacte bacterien van verschillende stammen, als met supernatant van een ultrasonicaat van deze bacterien, gaf een snelle isorhodopsine synthese te zien. De isomerisatie heeft alleen plaats in aanwezigheid van opsine. De feitelijke functie van het opsine in het isomerisatie proces en de identiteit van de bacteriele factor zijn nog niet bekend.

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STELLINGEN

I

De omstandigheden, gebruikt door Amer en Akhtar bij de bestudering van de rhodopsine regeneratie in vitro, zijn optimaal voor bacteriele isorhodopsine vorming.

S.Amer en M.Akhtar, Nature New Biology, 237, 266-267 (1972).

II

De bepaling van 11-cis retinaldehyde door middel van reactie met opsine is niet nauwkeurig, aangezien een variabele aspecifieke isomerisatie van het retinaldehyde optreedt.

R.Hubbard, P.K.Brown en D.Bownds, Meth. in Enzymol., 18C, p 243 (1972).

III

Het is misleidend de term rhodopsine regeneratie te gebruiken voor re-synthese van visueel pigment uit opsine en hetzij toegevoegd, hetzij door foto-isomerisatie verkregen 11-cis retinaldehyde.

H.Shichi, J.Biol.Chem., 246, 6178-6182 (1971).

IV

De experimenten van Cohen ondersteunen geenszins zijn conclusie dat een hoge mate van specificiteit van de m-RNA herkenningsfactoren in het KCl-extract aanwezig is.

B.B.Cohen, Biochim.Biophys.Acta, 247, 133-140 (1971).

V

Bij de bepaling van fosfodiesterase, zoals uitgevoerd door Jard et al, mag de hoeveelheid radio-actief cyclisch AMP niet verwaarloosd worden ten opzichte van de totale hoeveelheid substraat.

S.Jard en M.Bernard, Biochem.Biophys.Res.Comm., 41, 781-788 (1970).

VI

Bij de bereiding van het voor de bepaling van thyroxine (volgens Pileggi) benodigde broom-reagens, kan de optimale bromide/bromaat verhouding uit de reactievergelijking worden afgelezen.

V.J.Pileggi en G.Kessler, Clin.Chem., 14, 339-347 (1968).

J.A.Hathaway, Amer.J.Clin.Path., 53, 635-640 (1970).

A.Hanok, Amer.J.Clin.Path., 54, 542-561 (1970).

VII

Bij aerofiele bacteriën is de door Adler ontwikkelde methode voor het kwantitatief meten van chemotaxis niet zonder meer toepasbaar.

R.Mesibov en J.Adler, J.Bact., 112, 315-326 (1972).

G.L.Hazelbauer, R.E.Mesibov en J.Adler, Proc.Nat.Acad.Sci.(US), 64, 1300-1307 (1969).

VIII

De waarde van het thematisch interessante onderzoek van Bettelheim naar de aard van de kristalliniteit van een α-crystalline preparaat boet sterk aan waarde in door een reeks van onvolkomen- en onwaarschijnlijkheden.

F.A.Bettelheim, Exp.Eye Res., 14, 251-258 (1972).

IX

Het heeft weinig zin geluidsapparatuur te specificeren voor frequenties die voor het menselijk oor niet waarneembaar zijn.

J.P.Rotmans, 16 februari 1973.